Phylogeographic and morphometric studies on the Fischer's blue Tongeia fischeri (Lepidoptera: Lycaenidae) in Japan(Dissertation_全文)

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2013

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ABSTRACT

In this study, two different techniques and areas of scientific research in terms of genetic and morphological diversity were implemented to study the evolutionary history of the Fischer's blue *Tongeia fischeri* in Japan. First, a phylogeographical approach was used to understand the lineage diversity and phylogeographic pattern as well as demographic history of the butterfly. Second, a landmark-based geometric morphometric approach was used to examine morphological variations within and among the genetic lineages inferred by genetic data.

In the phylogeographic study, sequences of three mitochondrial genes (COI, Cyt b and ND5) and two nuclear genes (Rpl5 and Ldh) were used. Phylogenetic trees and the median-joining network revealed six evolutionary mitochondrial haplotype clades corresponding to geographic distribution of the species, including two clades from central Honshu, one clade from western Honshu and Shikoku, two clades from Kyushu and one clade from Tsushima Island. Divergence dates analysis revealed that common ancestors of Japanese *T. fischeri* might have come to Japan around 0.29 million years ago during the mid-Pleistocene by multiple dispersals of continental populations, probably via a land bridge or narrow channel between western Japan and the Korean Peninsula. Furthermore, geographical patterns of variation of mitochondrial and nuclear markers were discordant in northeastern Kyushu, possibly as a result of introgressive hybridization during the ancient contact between the Kyushu and Shikoku populations.

In the geometric morphometrics study, morphological variations in terms of wing size and shape variations between sexes and among genetically different populations were analyzed. Sexual dimorphism in wing size and shape was detected. Females had significantly larger wings than males, while males showed a relatively elongated forewing with a longer apex and a narrower wing tornus in comparison to females. Intraspecific variations in wing morphology among genetically different populations were revealed for the wing shape, but not the wing size. Phenotypic relationships inferred from wing-shape variation grouped *T. fischeri* populations into three groups, reflecting subspecies classification of the species. This study was the first to demonstrate the existence of wing morphological variation in *T. fischeri*, and to confirm morphological differentiation among genetically different populations. This study also revealed consistency between evolutionary relationships inferred from wing shape variation and the phylogenetic relationship obtained from molecular data, suggesting the presence of a phylogenetic signal in the wing shape of *T. fischeri*.

CHAPTER 1

Introduction

1.1 Structure of the thesis

In this thesis, I explore the genetic and morphological diversity of the Fischer's blue *Tongeia fischeri* (Eversmann, 1843) in Japan by means of phylogeographic and geometric morphometric approaches. In Chapter 1, a general introduction to the subject species, a brief description on the analytical approaches and the aims of this study are provided. In Chapter 2, I perform a phylogeographic analysis using mitochondrial and nuclear DNA markers, to discuss evolutionary history, demographic history and divergence time of the butterfly in Japan. In Chapter 3, I examine morphological variations of the wing of *T. fischeri* using landmark-based geometric morphometric approach and use the data to reconstruct the evolutionary relationship among *T. fischeri* populations. In the final chapter, Chapter 4, I discuss the main findings and future directions of my phylogeographic and morphometric studies on *T. fischeri*.

1.2 The Fischer's blue, *Tongeia fischeri*

Lycaenidae is a member of superfamily Papilionoidea (true butterfly). The family comprises of approximately 6000 species distributed worldwide and ranks the second-largest family of butterflies (Pierce *et al.*, 2002; Robbins, 1988). Butterflies in this family are commonly called as the gossamer winged butterflies, blues, coppers, or hairstreaks. The appeal of butterflies, particularly Lycaenidae, was attracted by scientists for a long time. Lycaenid butterflies typically have limited dispersal ability that strongly influenced by their hostplant distributions. They usually spend their adult life time taking a short flight foraging and mating around their hostplant, and commonly use only one species or one genus of plant (monophagus) (Fiedler, 1991). Moreover, more than a half of family members (75%) have been noted for an association with an ant at least one time on larval stage. The relationship between butterfly larvae and ants ranges from parasitism to mutualism or even predation (Fiedler, 1991; Pierce *et al.*, 2002). These properties enhance lyceanid butterflies to become an excellent subject for the study on evolution, speciation, ecology, and conservation biology (Lowe *et al.*, 2004).

The Fischer's blue *Tongeia fischeri* (Eversmann, 1843) is a small butterflies with approximate 2 cm of wingspan. The butterfly is characterized by a distinct black color on the uppersides of both forewing and hindwing, pale grey ground-color with three rows of black

dots and some additional black dots on the undersides of both wings, several orange tinged spots near the outer edge on the underside of the hindwing, and a pair of minute tails on the hindwing (Fig. 1-1). Males and females of *T. fischeri* are similar, but males are usually smaller than females. The butterfly is usually found in rocky steps, brook slopes of rivers, brook valleys, riverbanks, open forests, or coastal rocks, where their larval host plant grows (Fukuda *et al.*, 1984; Korshunov & Gorbunov, 1995). Adult of *T. fisheri* can be found in Japan from late April to early November with five broods in warm places and three broods in cold places (Fukuda *et al.*, 1984). Larvae of *T. fischeri* use hostplants in a couple genera of family Crassulaceae, mostly in the genus *Sedum* and *Orostachys* (Fukuda *et al.*, 1984; Korshunov & Gorbunov, 1995; Yakovlev, 2003). For Japanese populations, they were noted for using *Orostachys japonica* and *Sedum japonicum* subsp. *oryzifolium* as main hostplants, combining with some alternative hostplants including *Orostachys erubescens, O. iwarenge, O. aggreatus, Sedum sordidum, S. makinoi, S. tricarpum, S. lineare*, and *Hylotelephium sieboldi* (Fukuda *et al.*, 1984; Iwase, 1954). Almost all late instar larvae of *T. fischeri* are nearly permanently attended by ants (Fiedler, 1991; field observation). The relationship between *T. fischeri* larvae and ants is clearly facultative relationship, since the larvae are able to pupate and become adults successfully in the absence of ants under laboratory condition. Larvae of *T. fischeri* in Kyushu are noted to be tended by several species of ants, including *Lasius productus*, *Crematogaster nawai*, *Ochetellus glaber*, *Tetramorium tsushimae*, *T. bicarinatum*, *Pheidole fervens*, and *Cardiocondyla noda*, suggesting an unspecific host-usage of the butterfly (Jeratthitikul, unpublished data).

Tongeia fischeri has a wide distribution range covering almost all the part of East Asia, from southeast European Russia, southern Urals, Kazakhstan, the mountains of southern Siberia, Transbaikalia, southern Russia, Mongolia, northern China, Korea to Japan (Fig 1-2; Yakovlev, 2003). Among a total of 15 *Tongeia* species (Huang & Chen, 2006), *T. fisheri* seems to be one of most investigated member. Several taxonomic studies divided *T. fischeri* cumulatively into about 9 subspecies up-to-date (Fujioka, 1975; Hida, 2005; Huang & Chen, 2006; Satonaka, 2003; 2009; Shirôzu, 2006; Yago, 2007; Yakovlev, 2003). The Japanese populations are represented by three isolated populations, covering three of the main islands of Japan (Fig. 1-2; Biodiversity center of Japan, 2002). They were classified into three subspecies based on wing pattern and geographical distribution. Three subspecies are: (i) *T. f. japonica* Fujioka, 1975, occurring mainly in the Chubu district of central Honshu; (ii) *T. f. shojii* Satonaka, 2003, occurring in some parts of the Chugoku and Kinki districts of Honshu and the Shikoku district surrounding the Seto Inland sea, as well as in some parts of

eastern Fukuoka and Oita prefectures of the Kyushu district; and (iii) *T. f. shirozui* Hida, 2005, occurring in northern and western coastal Kyushu, including nearby islands such as Tsushima Islands and Yamaguchi prefecture in Honshu. However, Shirôzu (2006), Yago (2007), and Satonaka (2009) suggested that these populations of *T. f. shirozui* are included in *T. f.* caudalis, together with Korean and Chinese populations.

Although *T. fischeri* is still commonly found in some restricted localities, the overall numbers of butterflies in Japan have declined over the last decade. The reduction of the butterflies may be related to factory constructions, floods, host plant extinctions, urbanization, and/or ecological successions (Hirowatari, 1993). *Tongeia fischeri*, therefore, is designated as a near threatened species (NT) in the Red Data Book of the Ministry of the Environment, Japan (Yago, 2007).

1.3 Phylogeography

Phylogeography is a relatively new discipline that integrates phylogenetic analyses of organismal data with a geographical context. It is defined as a '… field of study concerned with the principles and processes governing the geographic distribution of genealogical lineages, especially within and among closely related species' (Avise, 2000). Phylogeographers interpret the extent and mode by which historical processes in population demographics have left evolutionary footprints on the contemporary geographic distributions of gene-based organismal traits, which may genetically structured due to environmental, geographical, geological, or behavioral factors (Avise, 2000; 2009; Avise *et al.*, 1987). The field of phylogeography has grown substantially since it was named in 1987 (Avise *et al.*, 1987) and still continues to increase in popularity, spanning usage to explain other kind of questions, such as ecological niche models, community assembly, and empirical systems (Hickerson *et al.*, 2010).

Mitochondrial DNA has been popular for animal phylogenetic and evolutionary study for a longtime. As a molecular marker, mitochondrial DNA possesses many advantages compared to those of nuclear DNA. It is easy to be isolated, since hundreds to thousands copies are presented in each animal cell. Mitochondrial DNA usually presents as a single haplotype in an individual, which can be read by the direct sequencing. It is also presumed lack of recombination in generally and uniparentally inherited. It has a generally higher mutation rate, although different regions on its genome evolve at different rates (Avise, 2000; Freeland, 2005). However, there are many arguments about the use of only mitochondrial DNA as a genetic marker in phylogenetic or phylogeographic studies in the recent years

(Galtier *et al.*, 2009; Hurst & Jiggins, 2005). The sole usage of mitochondrial DNA may yield an incomplete evolutionary history of the target species due to the indirect selection of mitochondrial DNA caused by symbionts (Hurst & Jiggins, 2005), mitochondrial recombination (Galtier *et al.*, 2009), and/or mitochondrial introgression (Gompert *et al.*, 2008; Kronforst *et al.*, 2006; Mallet, 2005). Therefore, recent, phylogeography has shifted toward the use of multilocus data of mitochondrial and nuclear DNA markers which allow researchers to answer more complicated evolutionary questions of target organisms (Zink & Barrowclough, 2008).

1.4 Geometric morphometrics

Morphometrics is a quantitative measurement analysis and comparing variability in size and shape of the biological morphological traits within and among groups of organisms (Reyment, 2010). Traditionally, morphometric data was measurements of linear distances (such as length, width, and height), ratios, masses, areas, or angles. However, such data set contains relatively little information about shape, and some of that information is fairly ambiguous, especially when the linear distance measurements are usually highly correlated with size (Zelditch *et al.*, 2004). More recently, a new morphometric approach that draws a precise and accurate description of shape variation has been developed (Zelditch *et al.*, 2004). 'Geometric morphometrics' is a multivariate statistical analysis of a biological shape using a set of landmark configurations to quantify biological shape, shape variation, and covariation of shape with other biotic or abiotic variables (Bookstein, 1996; Webster & Sheets, 2010; Zelditch *et al.*, 2004). Geometric morphometric can be divided into two types according to the nature of data acquisition: 'landmark-based geometric morphometrics' that summarizes shape in terms of a landmark configuration, and 'outline-based geometric morphometrics' that summarises the shape of open or closed curves, typically without fixed landmarks (Webster & Sheets, 2010).

Size measurements and the analysis of shape variation in geometric morphometrics depend on the selection of the set of 'landmarks', which can be defined as two or three-dimensional discrete anatomical points that can be recognized in all specimens of the study (Zelditch *et al.*, 2004). Because landmarks positions can convey importance of the study, landmark positions should be chosen carefully before commencing any morphometrics study. Wing venation provides an excellent landmark character for geometric morphometric study of butterflies and other winged insects (Breuker *et al.*, 2010). It suits well with five criteria of a good landmark listed by Zelditch *et al.* (2004). (i) Wing vein is homologous. (ii) It is easy

to identify and measure. As a flat structure, (iii) the placed landmarks are laid within the same plane. Vein network also covers the wing surface, thus (iv) provides an adequate coverage of the overall shape of the wing. Finally, each individual wing vein and inter vein area can be regulated independently from others, thus (v) each landmark has degree of independent from other landmarks in their location (Breuker *et al.*, 2010).

1.5 Aims

The pattern of the recent distribution and complexity of the morphological variation in *T. fischeri* enhances the butterfly as a good model for studying the mode of speciation and evolutionary history of the insect in Japan. In this study, two different techniques and areas of scientific research in terms of genetic and morphological diversity were implemented to study the evolutionary history of *T. fischeri* in Japan. First, a phylogeographical approach will explore several aspects of past and current distribution of the genetic lineages of *T. fischeri* in Japan. Second, a landmark-based geometric morphometric approach will examine the morphological variations between sexes and among the genetic lineages described by molecular markers.

The general aims of this study are:

1. To describe the lineage diversity and phylogeographic pattern as well as the demographic history of *T. fischeri* in Japan, using multilocus molecular markers
2. To estimate the morphological variability by means of wing size and shape variations between sexes and among genetically different populations of *T. fischeri* in Japan, using a geometric morphometric approach.
3. To compare evolutionary relationships among *T. fischeri* populations in Japan between that obtained from molecular and morphological data.

Figure legends

Figure 1-1. Photographs of *Tongeia fischeri*. Female upperside (A) and underside (B); Male upperside (C) and underside (D).

Figure 1-2. The distribution areas (solid lines) of *Tongeia fischeri* in the world (upper panel) and in Japan (lower panel). Distribution of *T. fischeri* in the world is modified from Yakovlev (2003) and in Japan from Biodiversity center of Japan (2002).

Figure 1-1

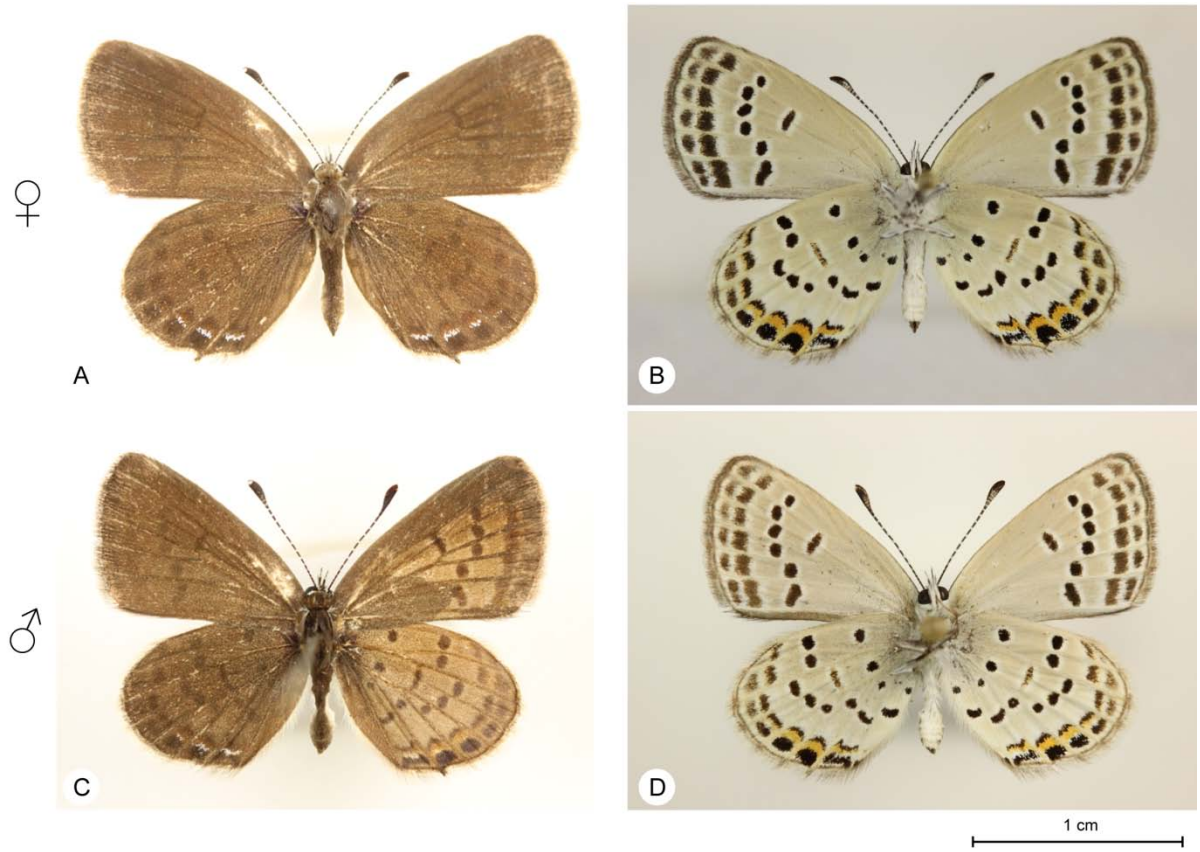
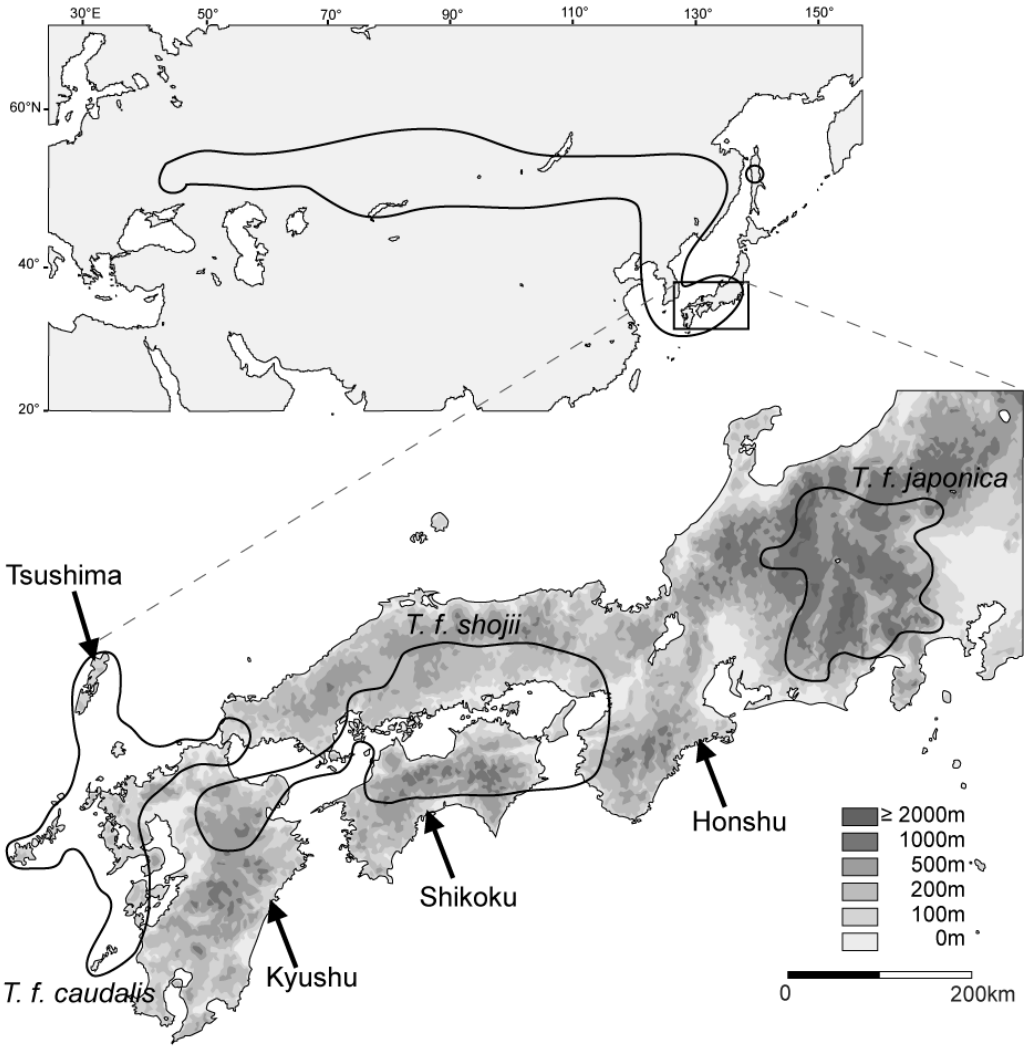


Figure 1-2


30°E
50°
70°
90°
110°
130°
150°
60°N
40°
20°
T. f. japonica
Tsushima
T. f. shojii
Honshu
Shikoku
Kyushu
T. f. caudalis
≥ 2000m
1000m
500m
200m
100m
0m
0
200km

CHAPTER 2

Phylogeography of Fischer's blue, Tongeia fischeri, in Japan: Evidence for introgressive hybridization

2.1 Abstract

The widespread lycaenid butterfly *Tongeia fischeri* is distributed from eastern Europe to northeastern Asia and represented by three geographically isolated populations in Japan. In order to clarify the phylogeographic history of the species, three mitochondrial (COI, Cyt b and ND5) and two nuclear (Rpl5 and Ldh) genes of 207 individuals collected from 55 sites throughout Japan and 5 sites on the Asian continent were sequenced. Phylogenetic trees and the median-joining network revealed six evolutionary mitochondrial haplotype clades, which corresponded to the geographic distribution of the species. Common ancestors of Japanese *T. fischeri* might have come to Japan during the mid-Pleistocene by multiple dispersals of continental populations, probably via a land bridge or narrow channel between western Japan and the Korean Peninsula. The geographical patterns of variation of mitochondrial and nuclear markers are discordant in northeastern Kyushu, possibly as a result of introgressive hybridization during the ancient contact between the Kyushu and Shikoku populations in the last glacial maximum. The phylogeographic pattern of *T. fischeri* in Japan are probably related to the geological history, Pleistocene climatic oscillations and distribution of the host plant.

2.2 Introduction

Geographic isolation between populations within a species has been noted to play an important role in allopatric speciation (Coyne & Orr, 2004; Sobel *et al.*, 2010). When gene flow has been limited or absent among populations, different or same alleles can become fixed depending upon the forces of natural selection and/or genetic drift in isolated populations. Thus, genetic divergence occurs over a long period, imparting reproductive isolation and possibly leading to the origin of new species (Coyne & Orr, 2004). If reproductive isolation is incomplete and formerly isolated lineages meet as a result of range expansion at a secondary contact zone, however, further mating between the populations will produce hybrids, resulting in the formation of a hybrid zone (Hewitt, 1988; 2001) genetic introgression between populations (Excoffier *et al.*, 2009) or reinforcement speciation (Coyne & Orr, 2004). These historical processes are expected to leave evolutionary evidences on the

distribution and frequency of alleles (Hewitt, 1996), and can be revealed by integration of phylogenetics and population genetics, as in the phylogeographic approach (Avise, 2000).

Phylogeography is a relative new discipline that deals with the spatial arrangement of genetic lineages, especially within and among closely related species. Phylogeographers interpret the extent to and mode by which historical processes in population demographics have left evolutionary footprints on the contemporary geographic distributions of gene-based organismal traits (Avise, 2000; 2009). Traditionally, phylogeographic studies relied on only mitochondrial DNA, which has been widely used in animal studies for a long time (Avise, 2000; Freeland, 2005). There are, however, many arguments about the use of only mitochondrial DNA as genetic markers in a phylogenetic or phylogeographic study that possibly leads to misinterpretation, for instance, due to the indirect selection caused by symbionts (Hurst & Jiggins, 2005), mitochondrial recombination in population levels (Galtier *et al.*, 2009) and mitochondrial introgression (Gompert *et al.*, 2008; Kronforst *et al.*, 2006; Mallet, 2005). The sole usage of mitochondrial DNA may yield an incomplete evolutionary history of the target species, whereas the combined use of mitochondrial and nuclear DNA markers allow researchers to answer more complicated evolutionary questions about target organisms (Zink & Barrowclough, 2008).

Fischer's blue, *Tongeia fischeri*, is a small butterfly (with an approximately 2.5 cm wingspan) in the family Lycaenidae. The butterfly is characterized by a distinct black color on the uppersides of both forewing and hindwing, pale grey ground-color with three rows of black dots and some additional black dots on the undersides of both wings, several orange tinged spots near the outer edge on the underside of the hindwing and a pair of minute tails on the hindwing. The butterfly is usually found in rocky steps, brook slopes of rivers, brook valleys, riverbanks, open forests or coastal rocks, where their larval host plant grows (Korshunov & Gorbunov, 1995). Figure 2-1 showed its wide distribution range covering southeastern European Russia, southern Urals, Kazakhstan, the mountains of southern Siberia, Transbaikalia, southern Russia, Mongolia, northern China, Korea and Japan at the eastern limit of the distribution (Yakovlev, 2003). Among a total of 15 described species of the genus *Tongeia*, *T. fischeri* seems to be one of the most widely investigated members (Huang & Chen, 2006). Several taxonomic studies divided *T. fischeri* cumulatively into about nine subspecies so far (Fujioka, 1975; Hida, 2005; Huang & Chen, 2006; Satonaka, 2003; 2009; Shirôzu, 2006; Yago, 2007; Yakovlev, 2003). The Japanese populations are represented by three isolated populations, covering three of the main islands of Japan (Fig. 2-1). They were classified into three subspecies based on the distinction of morphological traits

of the wing color and wing patterns: (i) the population occurring mainly in the Chubu district of central Honshu, named *T. f. japonica*, (ii) populations occurring in some parts of the Chugoku and Kinki districts of Honshu and the Shikoku district surrounding the Seto Inland sea, as well as in some parts of eastern Fukuoka and Oita prefectures of the Kyushu district, named *T. f. shojii*, and (iii) populations occurring in northern and western coastal Kyushu, including nearby islands such as Tsushima Is., and Yamaguchi prefecture in Honshu, named *T. f. shirozui*. However, Shirôzu(2006), Yago(2007) and Satonaka (2009) suggested that these populations are included in *T. f.* caudalis, together with Korean and Chinese populations.

Although *T. fischeri* is still commonly found in some restricted localities, the overall numbers of butterflies in Japan have declined over the last decade related to factory construction, floods, host plant extinction, urbanization and/or ecological successions (Hirowatari, 1993). Therefore, this species is designated as one of the near threatened species (NT) in the Red Data Book of the Ministry of the Environment, Japan (Yago, 2007).

The recent distribution pattern of *T. fischeri* in Japan and the complexity of its morphological variations provided a good model to study the mode of speciation and evolutionary history of insects in Japan. However, no molecular study of this species has been conducted hitherto. Here, mitochondrial and nuclear DNA sequences were collected. The evolutionary history of *T. fischeri* in Japan was then inferred using phylogenetic and phylogeographic approaches. Goals of this study are to describe the lineage diversity and phylogeographic pattern as well as the demographic history of *T. fischeri* in Japan.

2.3 Material and methods

2.3.1. Sampling

Two hundred and seven specimens of *T. fischeri* from 55 localities across the entire geographical range of the species in Japan and five localities on the Asian continent were collected (Fig. 2-1; Table 2-4). Three closely related species, *Tongeia hainani* and *T. filicaudis* from Taiwan, and *T. kala* from Myanmar were also used as the outgroups. Butterfly specimens were collected by hand netting in the field and immediately killed by pinching the thorax. Right wings (in some cases all wings) were removed and placed in triangular glassine envelopes with full data recorded for the vouchers. The butterfly bodies with or without other wings and abdomens were preserved with 100% ethanol and later stored at -80˚C for long-term storage at the Laboratory of Systematic Zoology, Kyoto University, and Faculty of Science, Shinshu University. In the case of dried museum specimens, the whole thorax was

removed from the butterfly body and then placed in a vials containing 100% ethanol for temporary storage prior to DNA extraction.

2.3.2. DNA extraction, amplification, sequencing and alignment

Total genomic DNA was extracted from a part of the thoracic muscle following the standard protocol of the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA, USA). Three protein-coding mitochondrial genes (mtDNA): Cytochrome oxidase subunit I (COI), Cytochrome b (Cytb) and NADH dehydrogenase subunit 5 (ND5), and two nuclear genes (nDNA): Ribosomal protein L5 gene (Rpl5) and Lactate dehydrogenase gene (Ldh), were used for molecular phylogenetic analysis. A fragment of Rpl5 contains two highly variable intron regions that useful for phylogenetic reconstruction (Mallarino *et al.*, 2005) and the Ldh contains one intron. One or two samples from each identical mitochondrial haplotype of each locality were selected for nuclear gene amplification. Such sampling strategy (e.g. Morando *et al.*, 2003) seems to give a reasonable result because individuals that have identical haplotype in more rapidly evolving gene (mitochondrial in comparison to nuclear gene) are expected to have similar or identical sequences in slowly evolving nuclear genes. However, this sampling strategy may yield a bias result when dealing with introgression, so all individuals in the introgression area (locality no. 40 and 46) were used for nuclear gene amplification. Gene fragments were amplified using polymerase chain reaction (PCR) in a MyCycler® thermal cycler (BIO-RAD) with the following thermal cycler conditions: 3 min at 94˚C followed by 35 cycles of 1 min at 94˚C, 1 min of annealing (case-by-case between 50˚C - 58˚C depending on the primer pair) and 150 sec at 72˚C, and finally extension at 72˚C for 5 min. In cases of specimens with poor DNA quality that were not amplified using published primers, internal-specific primers were designed from the alignment of the sequences in the successful cases. Primer names, references and primer sequences are presented in Table 2-1. The PCR products were purified by the PEG precipitation method and then sequenced on a 3130x1 Genetic Analyzer (Applied Biosystems) with the same primers used for amplification. The samples for which direct sequencing of the nuclear gene markers failed were subjected to subcloning using Promega pGEM-T Easy Vector System (Promega, Cat: A1360) to separate allelic sequences before sequencing.

Sequence alignment and editing were performed for each gene separately (COI, Cytb and ND5 were aligned together) by MEGA4 v4.0.2 (Tamura *et al.*, 2007). The three mitochondrial genes contained no gaps or indels, whereas Rpl5 and Ldh contained a few insertion/deletion indels. In these cases, automatic alignment in MEGA4 was performed and

then the alignments were edited manually. The alignments were determined for unique haplotypes using the software DNASP v4.0 (Rozas *et al.*, 2003) prior to subsequent analysis. Nucleotide sequences of unique haplotypes identified in this study have been deposited in the GenBank database under the GenBank ID: JQ423264 - JQ423458. GenBank accession numbers of haplotypes found for each locality are shown in Table 2-4.

The extents of genetic variation were estimated as the numbers of polymorphic (segregating) sites (*S*), average numbers of nucleotide differences (*k*), haplotype diversity (h: Nei, 1987) and nucleotide diversity (π: Nei & Li, 1979) using the software DNASP v4.0 (Rozas *et al.*, 2003).

2.3.3 Phylogenetic and haplotype networks analysis

Maximum Likelihood (ML) and Bayesian inference (BI) methods were used to reconstruct the phylogenetic relationship among haplotypes of mtDNA (COI, Cytb and ND5), nDNA (Rpl5, Ldh) and combined genes. The combined genes tree was reconstructed from 61 samples that were successfully amplified for all genes (the samples that have two nuclear alleles from difference clades were excluded). For mtDNA gene tree and combined gene tree reconstruction, dataset of each gene were set as partitions in the concatenated dataset with the evolutionary model specified for each partition separately.

Level of incongruence in phylogenetic reconstructions among mitochondrial genes partitions was assessed by comparing congruence and incongruence of well-supported clades across preliminary neighbor-joining and maximum parsimony trees of each gene partition. Preliminary trees were obtained from MEGA4 with 1000 bootstrap replications. In overall, the gene trees were not strongly conflict to each other and all well-supported clades were similar across all trees (data not shown). The incongruence length difference test (Farris *et al.*, 1995) was not performed because of its utility in evaluating homogeneity test still under debate (e.g. Barker & Lutzoni, 2002).

Best-fit models of nucleotide substitution as judged by the Akaike information criterion (AIC: Akaike, 1974) for ML and Bayesian information criterion (BIC: Schwarz, 1978) for BI were estimated in the program Kakusan4 (Tanabe, 2007; with maximum likelihoods calculated in Treefinder: Jobb et. al., 2004) before subsequently analyses. The ML analysis was performed on Treefinder (Jobb *et al.*, 2004), using the likelihood-ratchet method with 1000 bootstrap replicates to estimate branch confidence values. Tree topologies with bootstrap values (bs) 70% or greater were regarded as sufficiently resolved (Huelsenbeck & Hillis, 1993). BI analysis was performed with MrBayes v3.1 (Huelsenbeck

& Ronquist, 2001), which employs a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. A four-chain MC-MCMC analysis was run twice in parallel (with default heating values) for 5 million generations starting with random tree (10 million generations for combined genes dataset), and trees were sampled every 100 generations. The log-likelihood values of the sample points were plotted against the generation time, and 25% of the generations were discarded as "burn-in" samples. The remaining trees were used to estimate consensus tree topology, bipartition posterior probability (bpp) and branch length (Huelsenbeck & Ronquist, 2001). Bipartition posterior probability that was 0.95 or greater was considered as significantly supporting (Larget & Simon, 1999).

A median-joining network (Bandelt *et al.*, 1999) was also constructed for mtDNA dataset and two nDNA genes using the program Network (http://www.fluxus-technology.com). The median-joining method uses a maximum parsimony approach to search for all the shortest phylogenetic trees of given dataset (Bandelt *et al.*, 1999).

2.3.4 Estimation of divergence times

Divergence dates among the main mitochondrial clades were estimated using an uncorrelated lognormal relaxed clock in BEAST v1.6.1 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007). All individuals were used, not merely unique haplotypes, to avoid overestimates of evolutionary time scales (Marino *et al.*, 2011). Three mitochondrial genes (COI, Cytb and ND5) were used in this analysis. They were inputted separately with specified appropriate nucleotide substitution model selected by Kakusan4 under BIC. The coalescent exponential growth was chosen as a tree prior to avoid over parameterization of the model (Marino *et al.*, 2011) and a random tree generated from COI dataset was used as a start tree. A separate demographic model was applied following the approach of Ho et al. (2008). The exponential growth prior was restricted to ingroups and a normal prior was placed on the age of the root. Because of fossil data and geographical calibration event are not available. A universal arthropod mtDNA substitution rate of 2.3% divergence per million years (Brower, 1994) was used for tree dating. This substitution rate has been widely used for dating in insects (e.g. Lepidoptera: Parnassiinae, Nazari & Sperling, 2007). The use of substitution rates taken from literature without calibration points may give inappropriate results and should be interpreted with caution because of mitochondrial substitution rate usually vary across taxa and animal groups (Papadopoulou *et al.*, 2010 and reference therein). Two independent MCMC were run for 10 million generations and sampling every 1000th generation. The output files were checked for convergence diagnostics after removing a 10%

burn-in by examining Effective Sample Sizes (ESS) using Tracer v1.5 (Rambaut & Drummond, 2007). Results from two independent runs were pooled into a single combined result using LogCombiner v1.6.1 (in BEAST package) and then summarized in TreeAnnotator (in BEAST package) before visualizing the resulting tree in FigTree v1.3.1 (Rambaut, 2009). Time of the most recent common ancestor (tMRCA) for important nodes and main mitochondrial clades were reported as mean value of node height with 95% highest posterior density interval (HPD).

2.3.5 Historical demography

Demographic history of *T. fischeri* was revealed. Fu's *Fs* (Fu, 1997) and Tajima's *D* (Tajima, 1989) neutrality tests were employed as an assessment of possible population expansion by deviation from the neutrality. Large negative value of *Fs* reflects a numerous of rare alleles in a population, which indicates a recent increase in population size or positive selection. A significantly positive value of *D* can be interpreted as balancing selection, population subdivision or population contraction, while a significantly negative value indicates recent population size expansion (e.g. after a bottleneck or a selective sweep). Both statistics are expected to be close to zero in populations that have been stable over time (Fu, 1997; Tajima, 1989). These analyses were performed in ARLEQUIN v3.5 (Excoffier & Lischer, 2010) under 1000 bootstrap replicates for the significance test. In addition, Ramos-Onsins and Rozas' R_2 statistic (Ramos-Onsins & Rozas, 2002), which has more statistical power at small sample sizes, was calculated in DNASP v4.0 under 1000 coalescent simulations for the significance test. A significantly small positive value of R_2 reveals population growth. Analyses were conducted for all samples and on each haplotype clade suggested by phylogenetic analyses or the geographical region separately. Localities 40 and 46, where the sympatry of mitochondrial clades was evidenced (see results), were analyzed separately in mitochondrial dataset.

2.4 Results

2.4.1 Molecular data

Fragments of 1472 base pairs (bp) of COI (146 variable sites, 54 parsimony informative), 566 bp of Cytb (90 variable sites, 53 parsimony informative) and 433 bp of ND5 (49 variable sites, 16 parsimony informative) were obtained from all of 207 *T. fischeri* and three outgroups. For Rpl5, 68 sequences of 1307-1309 bp from 56 *T. fischeri* (12

specimens had heterozygous allele), one 1305 bp from *T. filicaudis* and one 1740 bp from *T.kala*, with 116 variable sites and 24 parsimony informative were obtained. For Ldh, 63 sequences of 762-771 bp from 48 *T. fischeri* (15 specimens had heterozygous allele) and one 746 bp from *T. kala*, with 88 variable sites and 17 parsimony informative were obtained. Ldh sequences of *T. hainani* and *T. filicaudis* and Rpl5 sequence of *T. hainani* were failed to amplify. Mitochondrial markers contained no insertions or deletions (indels) even when aligned with the outgroups, whereas Rpl5 and Ldh contained one and two indels, respectively. Pertaining to the ingroups, the percentage of parsimony-informative characters relative to the total characters for each gene were as follows: COI, 2.1%; Cytb, 1.9%; ND5, 2.0%; Rpl5, 1.7% and Ldh, 2.1%. Within the ingroups, mtDNA (COI, Cytb and ND5), Rpl5 and Ldh revealed, as a total, 78, 56 and 38 unique haplotypes, respectively.

Results of molecular diversity indices were given in Table 2-3. For each mitochondrial clade, the estimates of haplotype diversity (h) of almost all clades were high, ranging from 0.500 to 0.936, whereas nucleotide diversity(π) were relatively low, ranging from 0.00020 to 0.00284. For nuclear genes, overall variation across all samples in Ldh sequences was lower than those of Rpl5. Totally 38 different haplotypes out of 63 samples were identified from Ldh sequences (h = 0.912 and 58 segregating sites), whereas Rpl5 resulted 56 different haplotypes out of 68 samples (h = 0.986 and 99 segregating sites).

2.4.2 Phylogenetic analysis and haplotype networks

Gene genealogies estimated by ML and BI gave equivalent topologies for all genes. Only small differences of tip clades were detected, so that only tree topologies from BI analyses are shown in Fig. 2-2. The best-fit evolution models for the ML tree under AIC were: COI, GTR + G + I; Cytb, HKY85 + G; ND5, GTR + G + I; Rpl5, GTR + G and Ldh, K80; and for the BI tree under BIC were: COI, HKY + G + I; Cytb, HKY85 + G; ND5, GTR + G + I; Rpl5, HKY and Ldh, K80.

The phylogenetic tree from the mitochondrial data sets strongly supported monophyly of *T. fischeri* with an ML bootstrap value of 99.7%, and a Bayesian posterior probability of 1.00 (Fig. 2-2a). The mitochondrial tree revealed six geographically structured clades, including two well-supported sister clades from central Honshu (CH-I and CH-II), a western Honshu and Shikoku clade (WHS), a clade from western, northern and northeastern Kyushu (K-I), a clade from northwestern and northeastern Kyushu (K-II), and finally a clade from Tsushima Is. (TS). The clades from Honshu, CH-I and CH-II and WHS, formed a statistically supported monophyletic unit, whereas K-I, K-II and Ts clades were poorly supported and

placed at a basal part of the phylogenetic tree along with haplotypes from northern China, South Korea and Russia, which did not form a single clade. The distribution of mitochondrial clades on the map of Japan (Fig. 2-3a) clearly showed that these clades (distinguished by their colors) formed geographically separated clusters. However, clades K-I and K-II showed a region of sympatry in Oita and Fukuoka prefectures in northeastern Kyushu (localities 40 and 46 in Fig. 2-1). Furthermore, clade K-II included one haplotype (Mt70) from Tsushima Is. In addition, *T. filicaudis* and *T. hainani* were closer to *T. fischeri* than to *T. kala* among the three outgroups used in this study (Fig. 2-2a).

The Rpl5 data set reconstructed a phylogenetic tree similar to the mitochondrial one, but the Rpl5 tree showed less well-resolved topology in some points (Fig. 2-2b). The haplotypes were divided into three well-supported clades, Rpl5-I, Rpl5-II, and Rpl5-III. Rpl5-I included all of the mitochondrial clades CH-I and CH-II, while Rpl5-II corresponded to WHS and part of Kyushu clades K-I and K-II. Rpl5-III consisted of the rest of Kyushu clades K-I and K-II, TS and continental haplotypes. The distribution of the three Rpl5 clades was clearly divided into three geographically separated clusters, with one exception in northeastern Kyushu, where the Rpl5-II and the Rpl5-III clades exhibited a geographic overlap (Fig. 2-3b). Two samples from Oita Prefecture in northern Kyushu (locality 46 in Fig.2-1) had heterozygous alleles consisting of one from Rpl5-II and the other from Rpl5-III.

The Ldh data gave the lowest-resolution tree (Fig. 2-2c) with poorly supported haplotype groups: the Ldh-I clade (containing all the haplotypes of CH-I, CH-II, and WHS, and some haplotypes from eastern Kyushu of K-I and K-II), the Ldh-II clade composed of sub-clade Ldh-IIa (containing some haplotypes of K-I, K-II, some of TS, and a haplotype from northern China) and Ldh-IIb (a well-supported clade from Russia), and the Ldh-IIc clade (some of TS and haplotype Mt70 of the K-II clade). Like the Rpl5 gene, Ldh also showed a geographic overlap of clades in northeastern Kyushu (Fig. 2-3c), but no evidence of heterozygosity.

When all genes were combined, the phylogenetic tree was similar to the mitochondrial one, but the monophyly of K-I, K-II, Ts and continental haplotypes was revealed with strongly supported of ML bootstrap value of 94.1%, and a Bayesian posterior probability of 1.00 (Fig. 2-2d). Within this clade, TS was placed at the basal part of the clade and K-I and K-II were formed a statistically supported monophyletic unit. This result showed a clear relationship between *T. fisheri* in Japan which is helpfully in taxonomic classification.

The results from median-joining network analysis provided clearer resolution of the relationships among haplotypes (Fig. 2-3d, e, and f), and thus are more appropriate for

population level studies than phylogenetic trees. The mitochondrial and Rpl5 networks showed similar patterns, but the three main clades of Rpl5 were separated from each other more clearly by mutational steps and each cluster showed a star-like pattern (Fig. 2-3e). The Ldh networks showed some degree of discordance with the Ldh phylogenetic tree. Russian haplotypes (Ldh-IIb) were separated from the other two clusters by twelve mutational steps (Fig. 2-3f).

2.4.3. Estimation of divergence times

Divergence time analysis with an uncorrelated lognormal relaxed clock run in BEAST produced a tree with a topology similar to those of the combined gene dataset (Fig. 2-4), with some difference in subclade order. Mean ages and 95% highest posterior density (HPD) of mitochondrial clades are shown in Table 2-2. *Tongeia fischeri* diverged from its sister taxa around 1.68 (HPD = 1.12-3.30) million years ago (Mya) during the late Pliocene to early Pleistocene (node b in Fig. 2-4; Table 2-2). The ancestor of Japanese and continental *T. fischeri* further diverged around 0.29 Mya in the mid-Pleistocene (node d in Fig. 2-4; Table 2-2), corresponding to the cladogenetic event between Honshu (CH-I, CH-II and WHS clades), Kyushu (K-I, K-II), Tsushima Is. (TS) and continental populations. For the remaining major divisions thereafter, the divergence time might have been around the mid-Pleistocene to the last glacial maximum ranging from 0.19 to 0.02 Mya (nodes e-q in Fig. 2-4; Table 2-2).

2.4.4 Historical demography

Summary of population expansion test statistics are shown in Table 2-3. Considering *T. fischeri* as a single population, value of Fu's *Fs* was large negative and significant (-24.029), indicating a recent population expansion; however, negative value of Tajima's *D* and small positive value of Ramos-Onsins and Rozas' R_2 were not significant. Separate analyses strongly suggested a recent population expansion in CH-I and Ts with statistical significance in all statistics and K-II with significantly negative of *Fs*. Values of Tajima's *D* were positive for WHS, K-I, Northeastern Kyushu (locality no. 40 and 46), China and Russia and negative for CH-II and K-II, but all of them were not significant. The discordance between three approaches may due to the decrease of statistical power of Tajima's *D* in detecting significant change in population size and R_2 test is superior for small sample sizes, whereas *Fs* is better for large sample sizes (Ramos-Onsins & Rozas, 2002).

Two nuclear genes showed signs of population expansion evidenced by significant level of all statistical measures, negative *D*, large negative *Fs* and small positive R_2, in both all samples analysis and separate analysis for each clade, except for *Fs* in Ldh-II.

In summary, most of the major clades/regions of mitochondrial and two nuclear genes of *T. fishceri* in Japan showed signal of population expansion, although not always at a significant level. Only WHS, K-I, Northeastern Kyushu showed positive value of Tajima's *D*, indicating a rather structured and demographically stable population, although it was not significant.

2.5 Discussion

This study identified six mitochondrial clades within *T. fischeri* across its distribution range in Japan. These hypothetical clades seem to be restricted to within their respective geographical distribution ranges: no populations with the haplotype of one clade contained haplotype(s) of the other clades, and almost no haplotypes were shared among populations, with the exception of some populations in Kyushu and Tsushima Is. These results indicate that the gene flow among *T. fischeri* populations is highly limited, and the accumulation of mutations has led them to become evolutionarily distinct populations.

Although the pattern of differentiation in these evolutionarily distinct lineages was less clearly observed for both of the nuclear markers used, the distribution of nuclear haplotypes did not strongly conflict with that of the mitochondrial clades. The low resolution of the nuclear genealogy might be explained by the fact that nuclear genes show slower evolution compared to mitochondrial genes. Moore (1995) showed that a nuclear gene takes on average four times longer than a mitochondrial gene to form monophyly. In this study, nuclear gene genealogy failed to reveal a divergence among the Honshu clades (CH-I, CH-II and WHS) for the Ldh marker or between the Kyushu populations (K-I and K-II clades) for both nuclear markers. Such results suggested an incomplete lineage sorting of these nuclear markers within *T. fischeri* populations.

2.5.1 Phylogeographical pattern and evolutionary history of T. fischeri in Japan

Sota and Hayashi (2007) concluded that the colonization events of Japanese insects and land mammals occurred during the mid-Pleistocene, probably via a land bridge or narrow channel between western Japan and the East Asian continent during several regression periods during the glacial age. This also seems to have been the case for *T. fischeri*. The recent divergence of Japanese *T. fischeri* haplotypes suggested that their ancestor have came

to Japan around the mid-Pleistocene (0.29 Mya), as assumed from the oldest divergence time of the Japanese population. Thereafter, they became diverse and occupied the area of Japan under the influence of later Pleistocene events.

Subarctic coniferous forests expanded southward to lower land covering almost all areas of central Honshu around Lake Biwa (Kaizuka, 1980; Tsukada, 1983), especially during the last glacial maximum. Such habitat shifts of vegetation zones can result in limited distributions of herbivorous insects, such as butterflies (especially Lycaenidae), whose dispersal may depend on the host plant range. After *T. fischeri* occupied Honshu and expanded its distribution range northward along the island to the northern limit area, the continuous population might have suffered from Pleistocene climatic oscillations, which divided the common ancestor of the CH-I, CH-II and WHS clades into at least two refugia restricted to or near the present distribution range.

In the central Honshu population, CH-I and CH-II may have undergone allopatric fragmentation resulting in two mitochondrial haplotype lineages (which could not be detected by analysis of either of two slowly evolving nuclear genes): one of which, CH-I, is distributed in the areas to the east of Hida and Akaishi mountain ranges (dark green circles in Fig. 2-3a), while the other, CH-II, is a small haplotype group occurring in the valley between the Kiso and Akaishi mountain ranges in southern Nagano and northern Shizuoka (light green circles in Fig. 2-3a). The Akaishi mountain range is an important natural barrier to prevent gene flow between *T. fischeri* populations on the two sides of the mountain range. In addition, the effect of this barrier was probably much stronger during Pleistocene climatic oscillations. Butterfly populations might have been pushed to more favorable habitats and divided into sub-populations, and migrated by following the host plant shift to occupy lower altitude suitable habitat, and likely survived during the cool glacial periods in several refugia. Later, they expanded their population sizes when the climate became warmer. Such a pattern of recent population expansion was supported for CH-I clade by the significant of all demographic indices (Table 2-3) of the mitochondrial data set. This pattern has been reported before for the flightless beetle, *Silpha longicornis* (Ikeda *et al.*, 2009).

The WHS clade is distributed in western Honshu along the coast of the Seto Inland Sea, adjacent islands and Shikoku. A positive value of *D* and non-significance of *Fs* and R_2, suggested that the WHS clade was a genetically structured and demographically stable population, although we observed southward range expansion of WHS in the analyses of nuclear markers (which will be further discussed below).

Tongeia fischeri in Kyushu is distributed along the northern coast and neighboring northern edges of the eastern and western coasts as well as some adjacent islands. It is represented by two mitochondrial lineages (K-I and K-II) that occur sympatrically in the northeastern area. Although there is a geographic barrier (the sea) between Kyushu and the continent (Korean Peninsula), the shallow genetic divergence between the different continental populations and two Kyushu lineages, as in mtDNA tree, made to the hypothesis that Kyushu populations might have been diverged from different continental ancestors and by multiple dispersal events of the continental populations to Kyushu. According to the divergence time analysis (Fig. 2-4), the K-I lineage might have separated from Russian and Korean populations around 0.09 Mya, while K-II might have separated from TS. and Chinese lineage around 0.17 Mya. It could be hypothesized that the ancestor of K-II may have come to Kyushu first, and subsequently been followed by the K-I lineage. Alternatively, it is possible that the two lineages could have emerged as a result of one or a few dispersal events of a polymorphic ancestor, different haplotypes of which later somehow separately occupied different areas, and then later expanded their distribution ranges to overlap in northeastern Kyushu.

For the Tsushima clade (TS), the unique haplotypes might have become fixed after the separation from Kyushu and continental populations due to the rise of the sea level after the last glacial maximum. The haplotype sharing between southern Tsushima Is. and Kyushu was observed for the K-II mitochondrial clade (Mt70) and Ldh gene. Haplotype Mt70 is positioned near the root of the K-II clade of mitrochondrial network (Fig. 2-3d), and possibly represents the most ancestral allele in the K-II clade. This haplotype might provide evidence of the ancestral migration dispersal route of the Kyushu population from the continent. Alternatively, this apparent genetic contamination might have been caused by long distance-dispersal or artificial introduction from the northeastern Kyushu population to Tsushima Is.

The results further suggested that the Honshu population (CH-I, CH-II and WHS clades) might not be a descendent of the present Kyushu clades (K-I and/or K-II), but rather might have been derived independently from a different continental refugium. This hypothesis is supported by an allopatric divergence between the Kyushu and Honshu populations in the slowly evolving nuclear gene Ldh. Alternatively, this genetic break may correlated with the Bungo Strait in the Seto Inland Sea (Fig. 2-1). This strait originated during the late Pliocene to early Pleistocene, and since then has almost continuously separated Kyushu Island from Honshu and Shikoku Islands (Kaizuka, 1980). This geographical division also observed in many Japanese fauna, for example: the firefly, *Luciola*

cruciata (Suzuki *et al.*, 2002) and the bell-ring frog, *Buergeria buergeri* (Nishizawa *et al.*, 2011).

Additionally, the results did not show a pattern of postglacial northward range expansion from southern refugia. The common ancestors of *T. fischeri* might have been able to survive the cold climate during the last glacial maximum in areas relatively close to the present distribution areas, without moving to southern areas, although northward range expansion from southern refugia is commonly observed in European fauna (see review in Hewitt, 2011) and in some Japanese insects, such as the flightless beetle, *Carabus insulicola* (Sota & Nagata, 2008) and *Silphia perforate* (Ikeda *et al.*, 2009).

Larval host plant might have effects on the phylogeographical pattern of *T. fischeri* in Japan. Plants in the family Crassulaceae are known to be host plants for *T. fischeri* (Fukuda *et al.*, 1984; Korshunov & Gorbunov, 1995). Japanese populations use *Orostachys japonica* and *Sedum uniflorum oryzifolium* as their main host plants, in addition to several alternative host plants (Fukuda *et al.*, 1984). These two main host plants generally have distinct micro-habitats and distribution ranges: *O. japonica* grows naturally on sunny rocks in mountainous areas or on cliffs near rivers and the sea, and is distributed westward from the Kanto district in Honshu, Shikoku and Kyushu, whereas *S. uniflorum oryzifolium* grows along the seacoast, westward from the Kanto district on the Pacific Ocean side and westward from Fukui Prefecture on the Japan Sea side to Shikoku, Kyushu and the Ryukyus (Horikawa, 1976; Ohba, 2002) (sympatry of both species was observed in southern Tsushima Is., Fukuejima Is. and Kamikoshikijima Is. west of Kyushu, during the field research). Considering the distribution of the butterflies and the host plants, butterflies from central Honshu definitely use *O. japonica*, whereas populations in western Honshu, Shikoku and Kyushu generally use *O. japonica* where it grows, *S. uniflorum oryzifolium* in coastal areas, and both host plants in some areas, such as Fukuejima Is. and southern Tsushima Is. The results here are not consistent with ecological speciation of *T. fischeri* by host plants, although this phenomenon is often observed in other herbivorous insects (e.g. Nosil *et al.*, 2002; Ohshima & Yoshizawa, 2010). Nevertheless, the distributions of *T. fischeri* seem to be limited by their host plants' distribution. For example, the eastern limit in central Honshu, the restricted distribution in north and northeast Kyushu, and the absence of this butterfly around Lake Biwa in the Kinki district are probably related to lack of its host plant (Murata, 2004).

2.5.2 Introgressive hybridization in northeastern Kyushu

The geographical patterns of variation for mitochondrial and nuclear markers observed here were discordant. The major boundary between mitochondrial lineages corresponded to the Bungo strait, whereas some Honshu lineages of nuclear markers have crossed the strait and hybridized with Kyushu lineages in Fukuoka and Oita prefectures in northeastern Kyushu, as demonstrated by our phylogenetic trees and haplotype networks (Fig. 2-2 and 2-3). This phenomenon supposes to be the evidence for ancient hybridization followed by mitochondrial introgression. Although the connection between Honshu and Kyushu populations is limited by the water body in the Bungo strait at present, gene flow between them may possibly have occurred by the overlapping of expansion range during the land connection in the last glacial maximum (Kaizuka, 1980).

The spread of genes via hybridization from one population into the gene pool of another population is a relatively common phenomenon both above and below the species level (Gompert *et al.*, 2008; Kronforst *et al.*, 2006; Mallet, 2005), especially from the local resident species/population to the invading one after range expansion (Currat *et al.*, 2008; Excoffier *et al.*, 2009). In our case, one possibility is that the mitochondrial haplotypes from the Kyushu population were introduced into the expanding Shikoku population, and that once a Kyushu mitochondrial allele had entered the new population, the introgression might have been facilitated by selective sweeps (Maynard-Smith & Haigh, 1974) which could have led to the fixation of Kyushu haplotypes over Shikoku haplotypes, possibly due to direct and/or indirect positive selection (Ballard & Whitlock, 2004; Hurst & Jiggins, 2005) or without selection (Currat *et al.*, 2008). It is widely accepted that mitochondrial DNA can often be under selection, and this might be more common than previously thought (Ballard & Whitlock, 2004; Bensch *et al.*, 2006; Dowling *et al.*, 2008; Galtier *et al.*, 2009; Mishmar *et al.*, 2003). Alternatively, this observed pattern might have been driven by nuclear DNA introgression, but unlike the situation with mitochondrial markers, introduced nuclear markers are usually non-neutral and linked to other alleles with some deleterious effects in the novel genetic and external environment (Funk & Omland, 2003; Martinsen *et al.*, 2001). Although mitochondrial DNA introgression may be more likely than nuclear gene introgression (e.g. Ballard & Whitlock, 2004), cases of the latter still occur in nature, for example, in water flea, *Daphnia* spp. (Taylor *et al.*, 2005) and grasshopper, *Chorthippus parallelus* (Vazquez *et al.*, 1994). While the results of this study are consistent with the hypothesis of hybridization followed by mitochondrial introgression, specific selective pressures favoring Kyushu mitochondrial haplotypes were not clearly identified.

2.5.3 Taxonomic implications for T. fischeri

Using genetic results in this study as guide, especially in the combined genes analysis, the recognition of *T. f. japonica* in central Honshu (CH-I and CH-II clades) and *T. f. shojii* in western Honshu and Shikoku (WHS clade) is warranted. Kyushu populations (K-I, K-II and TS clades) as well as the Chinese, Korean and southern Russian populations should be noted as variant forms of *T. f. fischeri*. The populations in northeastern Kyushu, where the mitochondrial introgression has been observed, should be recognized as a hybrid population between *T. f. shojii* and *T. f. fischeri*. However, comprehensive analysis of morphological variations as well as phylogeographic study should be conducted with samples of all populations throughout the distribution range, especially with those from the continent, to confirm the subspecific taxonomy.

2.6 Conclusions

This study investigated the evolutionary history of *T. fischeri* throughout its distribution range in Japan. Six well-supported mitochondrial clades were recognized occupying specific habitats in three separate areas. Common ancestors of Japanese *T. fischeri* might have come to Japan during the mid-Pleistocene, probably via a land bridge or narrow channel between western Japan and the Korean Peninsula. The different clades most likely resulted from multiple immigration events at different times by continental populations dominated by different ancestral haplotypes, followed by further allopatric divergence. These phylogeographical patterns may have been triggered by the combination of geological history, distribution of host plant and climatic fluctuation during Pleistocene.

Figure legends

Figure 2-1. The distribution areas (solid lines) of *Tongeia fischeri* in the world (upper panel) and in Japan (lower panel). Sampling localities (open circles) are numbered referring to the locality names as shown in Table 2-4.

Figure 2-2. Phylogenetic relationships of *Tongeia fischeri* and the outgroups based on Bayesian inference analysis (BI) of mtDNA (a), Rpl5 (b), Ldh (c) genes and combined genes (d). Nodes with 0.95 or higher bipartition posterior probability for BI and/or 70% or higher bootstrap value for ML were regarded as sufficiently resolved nodes and marked with black circles (supported by both BI and ML), white circles (supported by BI) or grey circles (supported by ML), and are shown for some major nodes (ML/BI). Numbers in parentheses after haplotype name refer to sampling localities in Fig. 2-1 and list in Table 2-4.

Figure 2-3. Frequency distribution of hypothetical clades (left column) and median-joining networks (right column) for mtDNA (a, d), Rpl5 (b, e) and Ldh (c, f), with the size of circles proportional to sample sizes, and with colors and patterns following the tree hypothetical clades of genes in Fig. 2-2.

Figure 2-4. Bayesian Inference (BI) tree of mtDNA datasets for *T. fischeri* and the outgroups using uncorrelated lognormal relaxed clock in BEAST v1.6.1 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007), shows estimates of time since the most recent common ancestor (tMRCA) for each major node and main mitochondrial clades. Posterior probabilities of nodes are shown below node branch when higher than 0.95. Grey bars indicate 95% highest posterior density interval (HPD) of the node ages. Further details are found in Table 2-2.

Figure 2-1

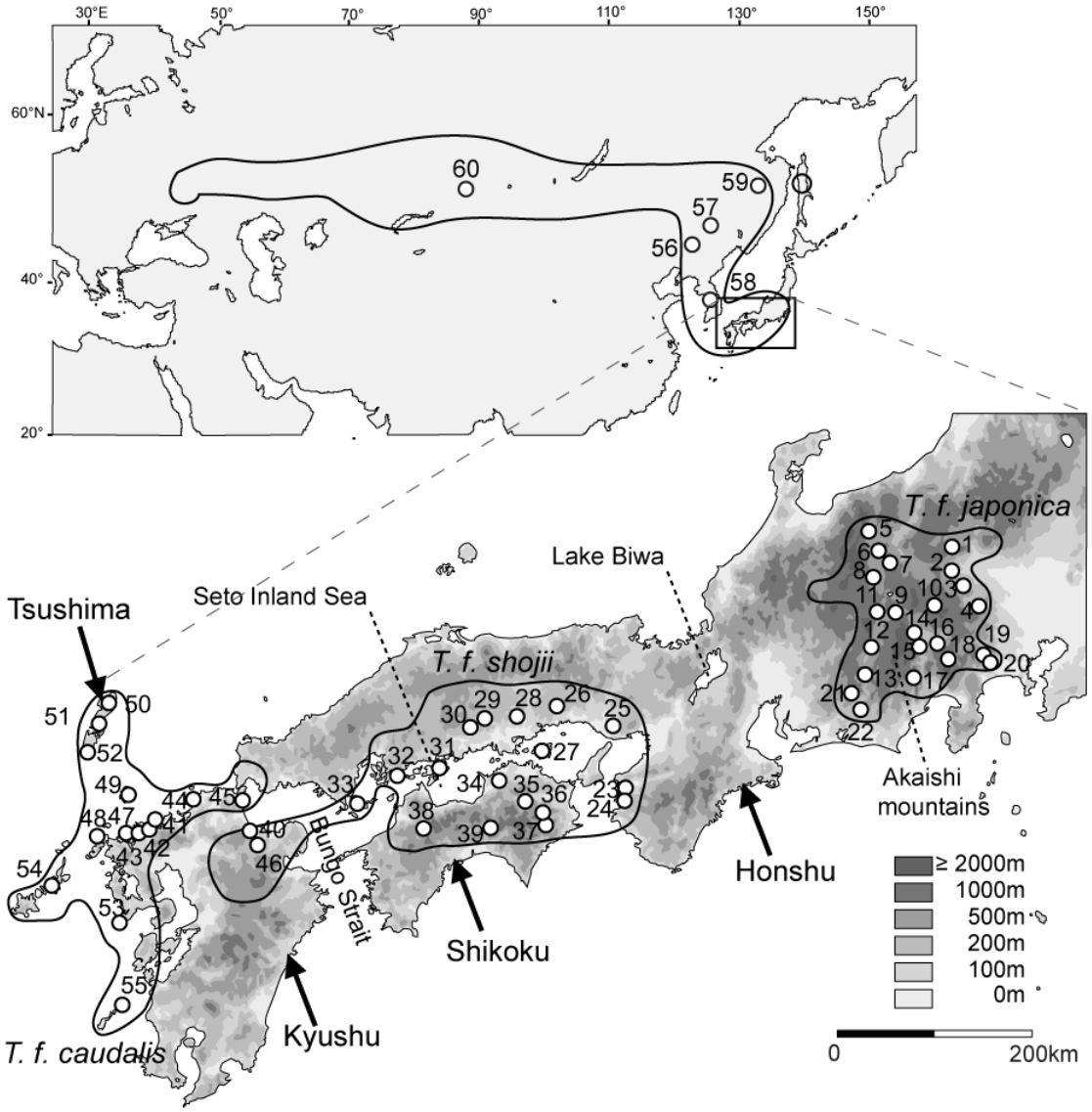

30°E
50°
70°
90°
110°
130°
150°
60°N
40°
20°
60
59
57
56
58
T. f. japonica
Lake Biwa
Seto Inland Sea
Tsushima
T. f. shojii
Akaishi
mountains
Honshu
Shikoku
Kyushu
Bungo Strait
T. f. caudalis
≥ 2000m
1000m
500m
200m
100m
0m
0
200km

Figure 2-2

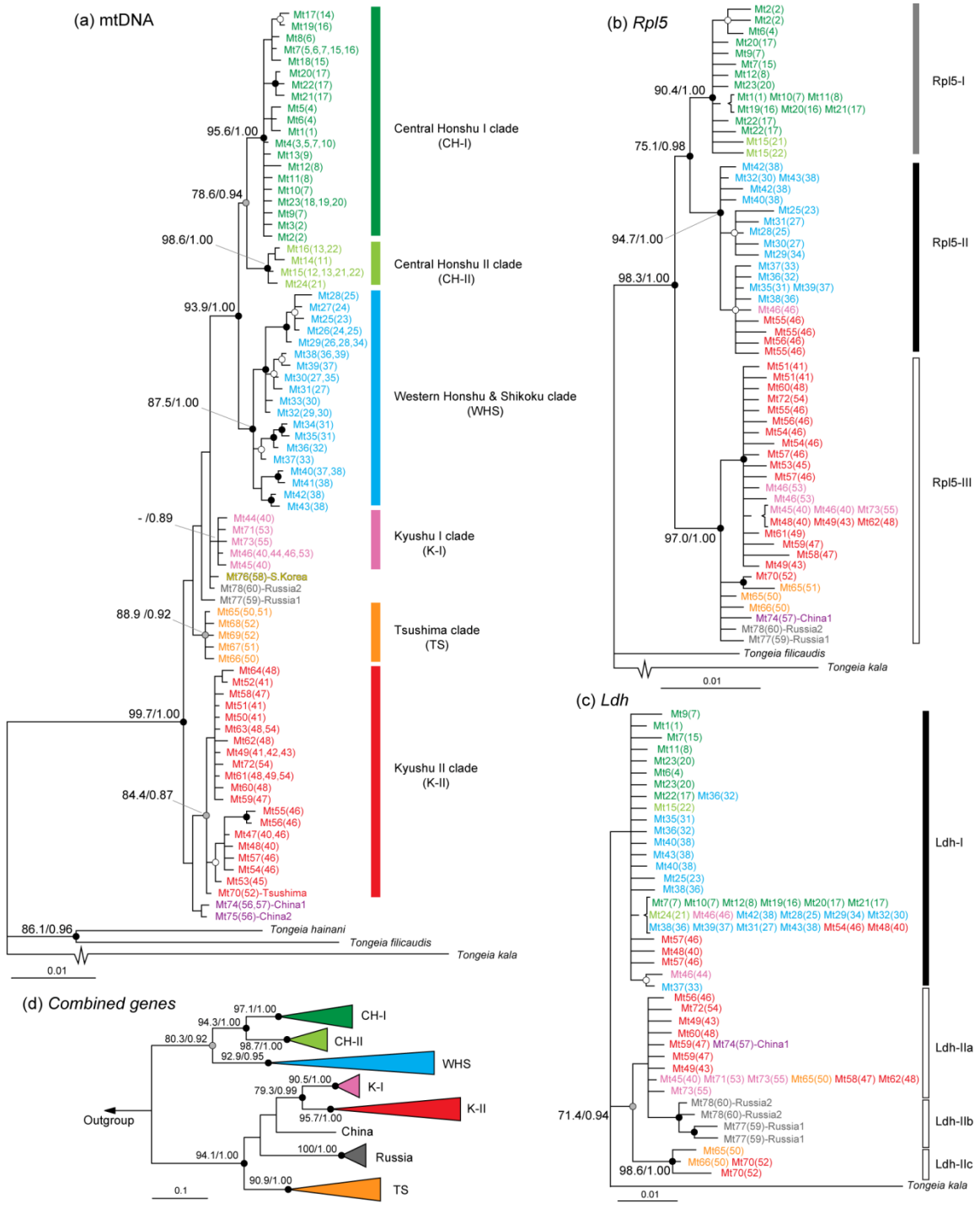

(a) mtDNA
Central Honshu I clade (CH-I)
Central Honshu II clade (CH-II)
Western Honshu & Shikoku clade (WHS)
Kyushu I clade (K-I)
Tsushima clade (TS)
Kyushu II clade (K-II)
Tongeia hainani
Tongeia filicaudis
Tongeia kala
(b) Rpl5
Rpl5-I
Rpl5-II
Rpl5-III
(c) Ldh
Ldh-I
Ldh-IIa
Ldh-IIb
Ldh-IIc
(d) Combined genes
CH-I
CH-II
WHS
K-I
K-II
China
Russia
TS
Outgroup

Figure 2-3

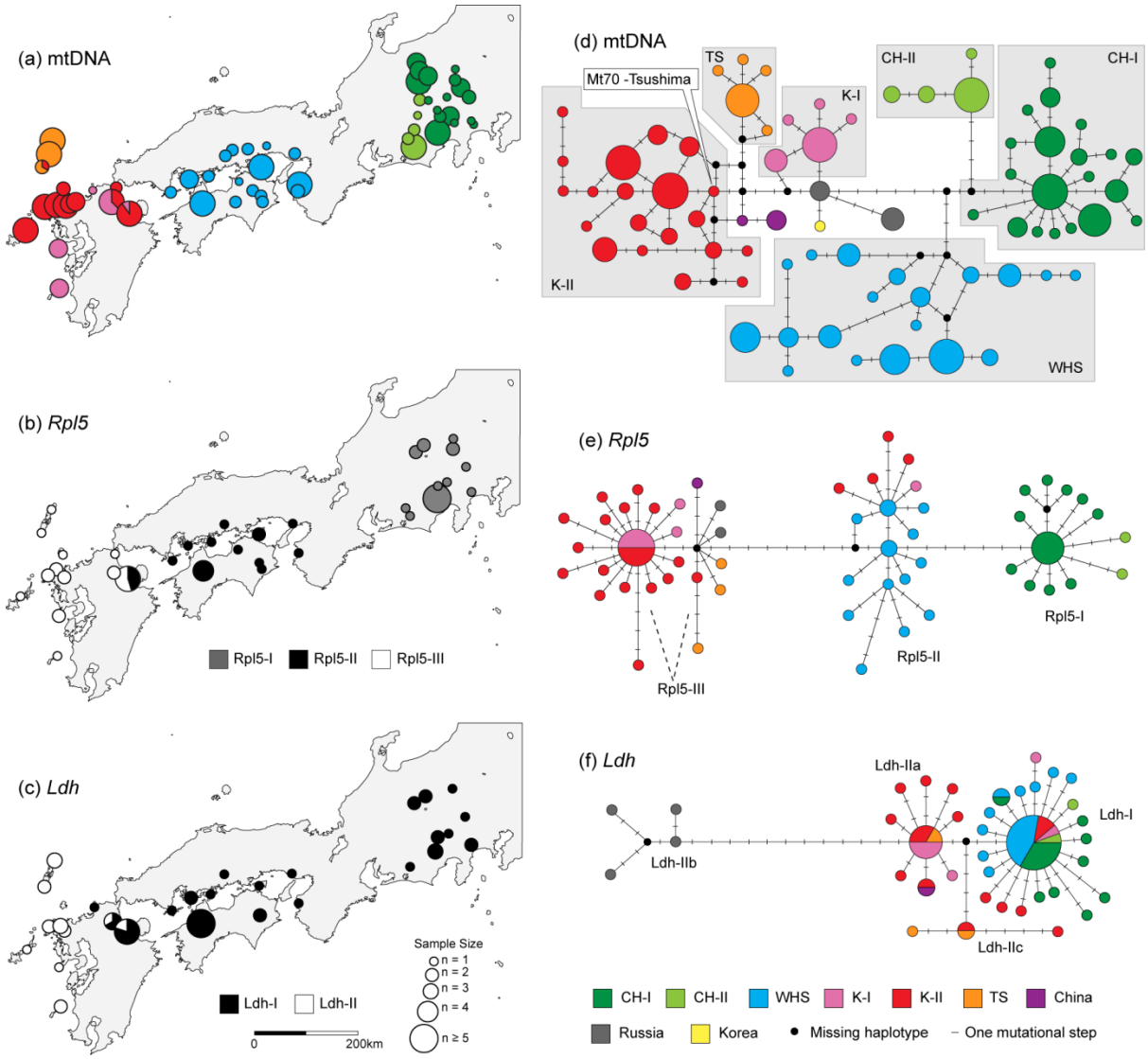

(a) mtDNA
(b) Rpl5
Rpl5-I
Rpl5-II
Rpl5-III
(c) Ldh
Ldh-I
Ldh-II
Sample Size
n = 1
n = 2
n = 3
n = 4
n ≥ 5
0
200km
(d) mtDNA
Mt70 -Tsushima
TS
K-I
CH-II
CH-I
K-II
WHS
(e) Rpl5
Rpl5-I
Rpl5-II
Rpl5-III
(f) Ldh
Ldh-IIa
Ldh-I
Ldh-IIb
Ldh-IIc
CH-I
CH-II
WHS
K-I
K-II
TS
China
Russia
Korea
Missing haplotype
One mutational step

Figure 2-4

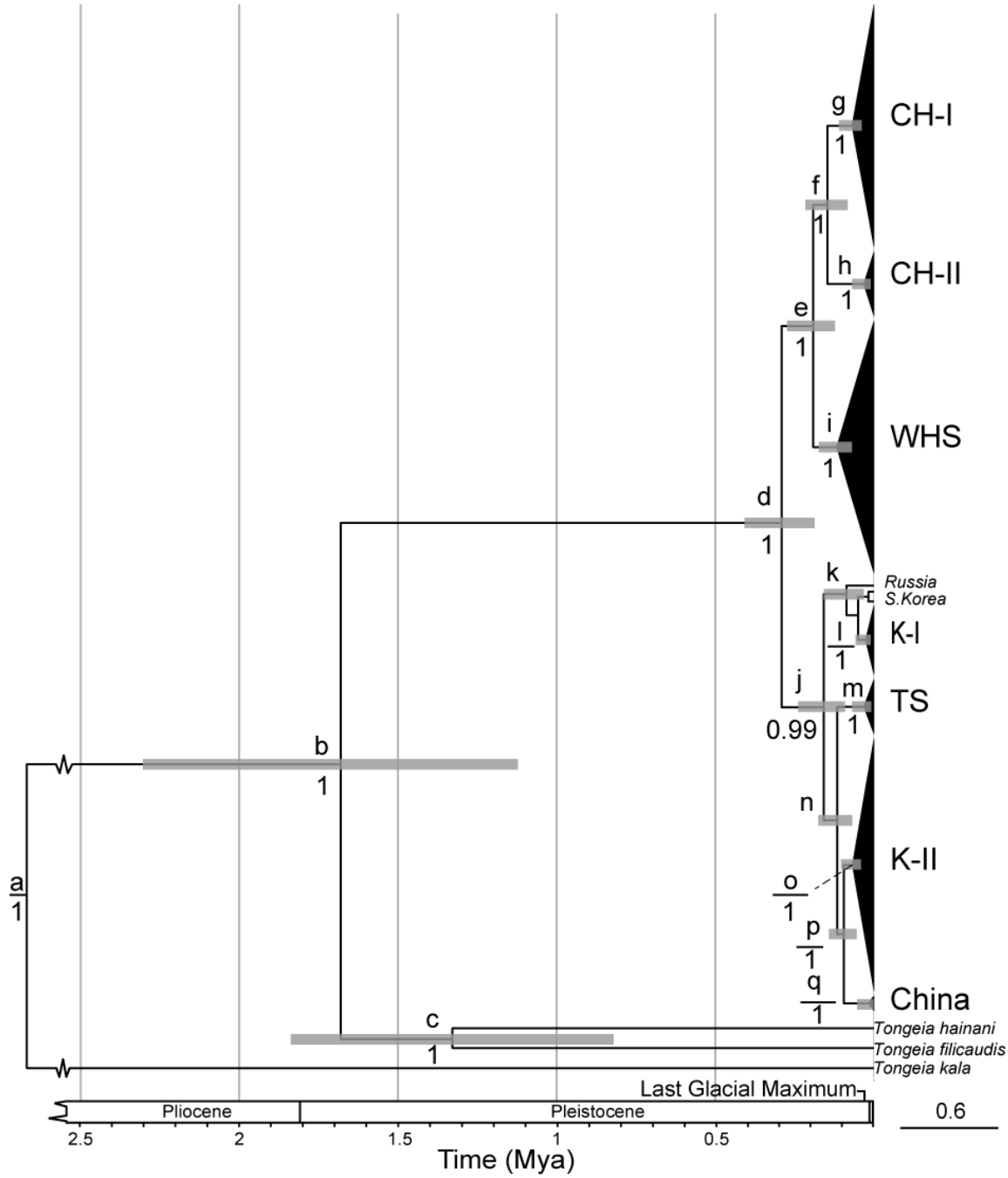

g
1
CH-I
f
1
h
1
CH-II
e
1
i
1
WHS
d
1
k
Russia
S.Korea
l
1
K-I
j
0.99
m
1
TS
b
1
n
K-II
a
1
o
1
p
1
q
1
China
c
1
Tongeia hainani
Tongeia filicaudis
Tongeia kala
Last Glacial Maximum
Pliocene
Pleistocene
0.6
2.5
2
1.5
1
0.5
Time (Mya)

Table 2-1. Primer sequences used in this study, those marked with '*' were specifically modified for this study. (F=Forward, R=Reverse)

Gene	Primer name	Reference	Sequence (5' to 3')
COI	LCO1490	Folmer et al. (1994)	GGTCAACAAATCATAAAGATATTGG (F)
	Nancy	Bogdanowicz et al. (1993)	CCCGGTAAAATTAAAATATAAACTTC (R)
	TN2126-TF*	Lohman et al. (2008)	TTGATCCTGCTGGAGGAGGTG (F)
	TF2758	This study	ATCGTCGAGGTATTCCAGCTAACC (R)
	TF2517	This study	CAGTAGAGGTTTAACAGGAG (F)
Cytb	TL2-N-3014	Simons et al. (1994)	TCCAATGCACTAATCTGCCATATTA (R)
	REVCB2H	Simmons and Weller (2001)	TGAGGACAAATATCATTTTGAGGW (F)
	REVCBJ	Simmons and Weller (2001)	ACTGGTCGAGCTCCAATTCATGT (R)
ND5	V1	Yagi et al. (1999)	CCTGTTTCTGCTTTAGTTCA (F)
	C2j	Yago et al. (2009)	CTAAAATTAWATCYTTAGARTAGAAYCC (R)
Rpl5	Rpl5-F 44	Mallarino et al. (2005)	TCCGACTTTCAAACAAGGATG (F)
	Rpl5-R441	Mallarino et al. (2005)	CATATCCTGGGAATCTCTTGATG (R)
Ldh	Ldhbm_65F	Dopman et al. (2005)	ATCGCCAGTAACCCCGTGG (F)
	Ldhbm_376R	Dopman et al. (2005)	CGATAGCCCAGGAAGTGTATCCCTTC (R)

Table 2-2. Estimated time since the most recent common ancestor (tMRCA) with 95% highest posterior density (HPD) of important divergence nodes and mitochondrial haplotype clades. Nodes correspond to 17 labels in Fig. 2-4. Time scale in million years ago (Mya).

Node	Clade	tMRCA (95%HPD)
a	All (*T. fischeri* and outgroups)	5.45 (3.43-8.14)
b	*T. fischeri*, *T. hainani* and *T. filicaudis*	1.68(1.12-2.30)
c	*T. hainani* and *T. filicaudis*	1.33 (0.82-1.18)
d	*T. fischeri*	0.29 (0.19-0.41)
e	Honshu (CH-I, CH-II and WHS)	0.19 (0.12-0.27)
f	Central Honshu (CH-I and CH-II)	0.15 (0.08-0.22)
g	CH-I	0.07 (0.04-0.11)
h	CH-II	0.03 (0.01-0.07)
i	WHS	0.12 (0.07-0.17)
j	Kyushu (K-I and K-II), Tsushima (TS) and Continent	0.16 (0.09-0.24)
k	K-I, Russia and S.Korea	0.09 (0.03-0.16)
l	K-I	0.03 (0.01-0.06)
m	K-II , Ts and China	0.17 (0.07-0.18)
n	TS	0.03 (0.01-0.07)
o	K-II and China	0.09 (0.06-0.14)
p	K-II	0.07 (0.04-0.10)
q	China	0.02 (0.00-0.05)

Table 2-3. Summary of molecular diversity indices and population expansion test statistics of mitochondrial (combined for COI, Cytb and ND5), Rpl5 and Ldh genes; sample size (n), number of haplotypes (No.), number of polymorphic (segregating) sites (*S*), average number of nucleotide differences (*k*), haplotype diversity (*h*) and nucleotide diversity (π) with standard deviation (SD), Tajima's *D*, Fu's *Fs* and Ramos-Onsins and Rozas' R_2.

Gene	Clade/Region	n	No.	*S*	*k*	*h* (± SD)	π (± SD)	*D*	*Fs*	R_2
mtDNA	CH-I	50	20	21	2.211	0.919(0.020)	0.00090(0.00008)	-1.695*	-13.616**	0.049*
	CH-II	14	4	3	0.846	0.582(0.137)	0.00034(0.00010)	-0.315	-0.784	0.145
	WHS	52	19	23	5.613	0.936 (0.014)	0.00228(0.00015)	0.331	-3.11	0.12
	K-I (locality no. 40 and 46 excluded)	9	3	2	0.778	0.667(0.105)	0.00032(0.00007)	0.196	-0.108	0.21
	K-II (locality no. 40 and 46 excluded)	40	14	10	1.867	0.872(0.034)	0.00076(0.00010)	-0.614	-7.385**	0.092
	Northeastern Kyushu (locality no. 40 and 46)	18	9	18	6.993	0.882(0.051)	0.00284(0.00024)	1.299	0.638	0.186
	TS	12	5	4	0.667	0.576(0.163)	0.00027(0.00009)	-1.747*	-2.980**	0.118**
	S.Korea	1	1	/	/	/	/	/	/	/
	China	4	2	1	0.5	0.500(0.265)	0.00020(0.00011)	1.633	0.54	0.433
	Russia	7	2	3	1.714	0.571(0.119)	0.00070(0.00015)	1.811	2.92	0.077
	All samples	207	78	72	11.31	0.981(0.002)	0.00460(0.00008)	-0.167	-24.029**	0.077
Rpl5	Rpl5-I	18	13	21	2.516	0.902(0.066)	0.00193(0.00036)	-2.302**	-8.950**	0.048**
	Rpl5-II	20	18	27	3.605	0.989(0.019)	0.00275(0.00036)	-2.120**	-13.710**	0.044**
	Rpl5-III	30	25	44	3.789	0.966(0.027)	0.00289(0.00039)	-2.506**	-21.265**	0.026**
	All samples	68	56	99	9.105	0.986(0.008)	0.00697(0.00023)	-1.917**	-24.589**	0.036**
Ldh	Ldh-I	40	22	29	1.594	0.803(0.067)	0.00210(0.00034)	-2.630**	-24.945**	0.030**
	Ldh-II	23	16	31	4.561	0.933(0.042)	0.00599(0.00105)	-1.742*	-4.186	0.056**
	All samples	63	38	58	3.59	0.912(0.030)	0.00472(0.00056)	-2.297**	-25.475**	0.024**

*P<0.05; **P<0.01

Table 2-4. *Tongeia fischeri* specimens examined, location number (No.), taxon, locality name, approximate coordinates, samples size (*N*), sample storage method, mitochondrial haplotype name with the corresponding sample size (*N*) when it is other than one, and GenBank accession numbers of mitochondrial genes and nuclear genes analyzed in this study.

No.	Taxon	Locality name	Approximate Coordinates	*N*	Sample Storage	mtDNA Haplotypes	GenBank ID				
							COI	Cytb	ND5	Rpl5	Ldh
1	*T. fischeri*	Agatsuma, Gunma	36.53 N 138.76 E	1	ETOH	Mt1	JQ423267	JQ423312	JQ423338	JQ423419	JQ423364
2	*T. fischeri*	Kanra, Gunma	36.20 N 138.69 E	2	ETOH/ Pinned	Mt2, Mt3	JQ423268, JQ423269	JQ423312	JQ423339	JQ423430, JQ423431	-
3	*T. fischeri*	Tomioka, Gunma	36.26 N 138.89 E	4	ETOH	Mt4(4)	JQ423270	JQ423312	JQ423339	-	-
4	*T. fischeri*	Chichibu, Saitama	36.02 N 138.93 E	3	ETOH/ Pinned	Mt5, Mt6(2)	JQ423270	JQ423312	JQ423338	JQ423429	JQ423375
5	*T. fischeri*	Kitaazumi, Nagano	36.70 N 137.84 E	4	ETOH	Mt4(3), Mt7	JQ423270, JQ423271	JQ423312	JQ423339, JQ423340	-	-
6	*T. fischeri*	Omachi, Nagano	36.61 N 137.85 E	4	ETOH	Mt7(3) ,Mt8	JQ423271	JQ423312	JQ423339, JQ423341	-	-
7	*T. fischeri*	Ueda, Nagano	36.28 N 138.23 E	4	ETOH	Mt4, Mt7, Mt9, Mt10	JQ423270, JQ423271, JQ423272	JQ423312, JQ423313	JQ423339	JQ423419, JQ423423	JQ423363, JQ423379
8	*T. fischeri*	Matsumoto, Nagano	36.25 N 137.96 E	9	ETOH	Mt11(8), Mt12	JQ423273, JQ423274	JQ423312, JQ423314	JQ423339	JQ423419, JQ423421	JQ423379, JQ423381
9	*T. fischeri*	Ina, Nagano	35.86 N 137.95 E	1	ETOH	Mt13	JQ423270	JQ423312	JQ423341	-	-
10	*T. fischeri*	Minamisaku, Nagano	35.94 N 138.62 E	1	ETOH	Mt4	JQ423270	JQ423312	JQ423339	-	-
11	*T. fischeri*	Komagane, Nagano	35.74 N 137.97 E	2	ETOH	Mt14(2)	JQ423277	JQ423320	JQ423342	-	-
12	*T. fischeri*	Ida, Nagano	35.40 N 137.97 E	1	ETOH	Mt15	JQ423277	JQ423320	JQ423343	-	-
13	*T. fischeri*	Shimoina, Nagano	35.34 N 137.73 E	2	ETOH	Mt15, Mt16	JQ423277	JQ423320	JQ423343, JQ423344	-	-
14	*T. fischeri*	Hokuto, Yamanashi	35.78 N 138.43 E	1	Pinned	Mt17	JQ423271	JQ423315	JQ423339	-	-
15	*T. fischeri*	Minami-Alps, Yamanashi	35.61 N 138.46 E	2	Pinned	Mt7, Mt18	JQ423271, JQ423275	JQ423312	JQ423339	JQ423426	JQ423369, JQ423379
16	*T. fischeri*	Nirasaki, Yamanashi	35.70 N 138.45 E	4	ETOH/ Pinned	Mt7, Mt19(3)	JQ423271	JQ423312, JQ423316	JQ423339	JQ423419	JQ423379
17	*T. fischeri*	Minamikoma, Yamanashi	35.37 N 138.40 E	7	ETOH	Mt20(4), Mt21, Mt22(2)	JQ423276	JQ423312, JQ423317,	JQ423339	JQ423419, JQ423420,	JQ423378, JQ423379

No.	Taxon	Locality name	Approximate Coordinates	*N*	Sample Storage	mtDNA Haplotypes	GenBank ID				
							COI	Cytb	ND5	Rpl5	Ldh
								JQ423318		JQ423424, JQ423425	
18	*T. fischeri*	Otsuki, Yamanashi	35.62 N 138.95 E	1	ETOH	Mt23	JQ423270	JQ423319	JQ423339	-	-
19	*T. fischeri*	Tsukui, Kanagawa	35.58 N 139.27 E	1	ETOH	Mt23	JQ423270	JQ423319	JQ423339	-	-
20	*T. fischeri*	Sagamihara, Kanagawa	35.62 N 139.19 E	1	Pinned	Mt23	JQ423270	JQ423319	JQ423339	JQ423422	JQ423374, JQ423376
21	*T. fischeri*	Kitashitara, Aichi	35.20 N 137.78 E	2	ETOH	Mt15, Mt24	JQ423277, JQ423278	JQ423320	JQ423343	JQ423427	JQ423379
22	*T. fischeri*	Hamamatsu, Shizuoka	34.88 N 137.82 E	7	ETOH	Mt15(6), Mt16	JQ423277	JQ423320	JQ423343, JQ423344	JQ423428	JQ423372
23	*T. fischeri*	Wakayama, Wakayama	34.25 N 135.16 E	6	Pinned	Mt25(6)	JQ423279	JQ423321	JQ423338	JQ423417	JQ423367
24	*T. fischeri*	Kainan, Wakayama	34.17 N 135.21 E	3	ETOH	Mt26(2), Mt27	JQ423279	JQ423321	JQ423339, JQ423341	-	-
25	*T. fischeri*	Kobe, Hyogo	34.77 N 135.19 E	2	ETOH	Mt26, Mt28	JQ423279	JQ423321, JQ423322	JQ423339	JQ423414	JQ423379
26	*T. fischeri*	Shiso, Hyogo	35.01 N 134.55 E	1	ETOH	Mt29	JQ423279	JQ423323	JQ423339	-	-
27	*T. fischeri*	Shodoshima Is., Kagawa	34.50 N 134.30 E	8	ETOH	Mt30(6), Mt31(2)	JQ423280, JQ423281	JQ423323	JQ423339	JQ423416, JQ423418	JQ423379
28	*T. fischeri*	Wake, Okayama	34.80 N 134.15 E	2	ETOH	Mt29(2)	JQ423279	JQ423323	JQ423339	-	-
29	*T. fischeri*	Takahashi, Okayama	34.79 N 133.53 E	2	Pinned	Mt32(2)	JQ423282	JQ423323	JQ423339	-	-
30	*T. fischeri*	Kawakami, Okayama	34.84 N 133.36 E	2	ETOH	Mt32, Mt33	JQ423282	JQ423323	JQ423339, JQ423341	JQ423412	JQ423379
31	*T. fischeri*	Innoshima Is., Hiroshima	34.32 N 133.17 E	2	ETOH	Mt34, Mt35	JQ423283	JQ423324	JQ423339, JQ423341	JQ423406	JQ423373
32	*T. fischeri*	Kamagarijima Is.., Hiroshima	34.19N 132.69 E	4	ETOH	Mt36(4)	JQ423284	JQ423324	JQ423339	JQ423405	JQ423377, JQ423378
33	*T. fischeri*	Yashirojima Is., Yamaguchi	33.93 N 132.22 E	2	ETOH	Mt37(2)	JQ423285	JQ423325	JQ423339	JQ423410	JQ423362
34	*T. fischeri*	Mitoyo, Kagawa	34.20 N 133.74 E	1	ETOH	Mt29	JQ423279	JQ423323	JQ423339	JQ423415	JQ423379
35	*T. fischeri*	Mima, Tokushima	34.06 N 134.12 E	2	Pinned	Mt30(2)	JQ423280	JQ423323	JQ423339	-	-
36	*T. fischeri*	Kitoumura, Tokushima	33.77 N 134.20 E	2	ETOH	Mt38(2)	JQ423286	JQ423323	JQ423339	JQ423403	JQ423368, JQ423379
37	*T. fischeri*	Naka, Tokushima	33.81 N 134.37 E	3	ETOH	H39, Mt40(2)	JQ423286, JQ423287	JQ423323	JQ423339	JQ423406	JQ423379

No.	Taxon	Locality name	Approximate Coordinates	*N*	Sample Storage	mtDNA Haplotypes	GenBank ID				
							COI	Cytb	ND5	Rpl5	Ldh
38	*T. fischeri*	Kamiukena, Ehime	33.59 N 132.99 E	8	ETOH	Mt40(4), Mt41, Mt42(2), Mt43	JQ423288, JQ423289	JQ423325, JQ423326, JQ423327	JQ423339, JQ423345, JQ423346	JQ423408, JQ423411, JQ423412, JQ423413	JQ423365, JQ423371, JQ423379, JQ423382
39	*T. fischeri*	Nagaoka, Kochi	33.82 N 131.15 E	2	Pinned	Mt38(2)	JQ423286	JQ423323	JQ423339	-	-
40	*T. fischeri*	Chikujou, Fukuoka	33.54 N 131.15 E	8	ETOH	Mt44, Mt45, Mt46(3), Mt47, Mt48(2)	JQ423290, JQ423291, JQ423293	JQ423328	JQ423347, JQ423348, JQ423350, JQ423351	JQ423441	JQ423370, JQ423379, JQ423393
41	*T. fischeri*	Fukuoka, Fukuoka	33.58 N 130.26 E	4	ETOH	Mt49, Mt50, Mt51, Mt52	JQ423294	JQ423329	JQ423352, JQ423353, JQ423354, JQ423355	JQ423444, JQ423454	-
42	*T. fischeri*	Itoshima, Fukuoka	33.56 N 130.18 E	4	ETOH	Mt49(4)	JQ423294	JQ423329	JQ423352	-	-
43	*T. fischeri*	Himeshima Is., Fukuoka	33.57 N 130.05 E	5	ETOH	Mt49(5)	JQ423294	JQ423329	JQ423352	JQ423441, JQ423453	JQ423395, JQ423399
44	*T. fischeri*	Ainoshima Is., Fukuoka,.	33.76 N 130.37 E	1	Pinned	Mt46	JQ423291	JQ423328	JQ423347	-	JQ423361
45	*T. fischeri*	Kitakyushu, Fukuoka	33.91 N 130.99 E	2		Mt53(2)	JQ423293	JQ423328	JQ423352	JQ423449	-
46	*T. fischeri*	Nakatsu, Oita	33.46 N 131.18 E	10	ETOH/ Pinned	Mt46, Mt47, Mt54, Mt55(5), Mt56, Mt57	JQ423291, JQ423293, JQ423295, JQ423296	JQ423328, JQ423330, JQ423331, JQ423332	JQ423347, JQ423350, JQ423356	JQ423401, JQ423402, JQ423404, JQ423407, JQ423409, JQ423442, JQ423446, JQ423451, JQ423452, JQ423455, JQ423456	JQ423366, JQ423379, JQ423380, JQ423383
47	*T. fischeri*	Karatsu, Saga	33.52 N 129.86 E	5	ETOH	Mt58(3), Mt59(2)	JQ423297, JQ423298	JQ423329, JQ423333	JQ423352	JQ423439, JQ423450	JQ423384, JQ423393, JQ423397
48	*T. fischeri*	Hirado, Nagasaki	33.38 N 129.42 E	8	ETOH	Mt60(2), Mt61(3),	JQ423298, JQ423299	JQ423329, JQ423334	JQ423352, JQ423353,	JQ423441, JQ423445	JQ423389, JQ423398

No.	Taxon	Locality name	Approximate Coordinates	*N*	Sample Storage	mtDNA Haplotypes	GenBank ID				
							COI	Cytb	ND5	Rpl5	Ldh
						Mt62,Mt63, Mt64			JQ423356, JQ423357		
49	*T. fischeri*	Ikinoshima Is., Nagasaki	33.78 N 129.73 E	3	ETOH	Mt61(3)	JQ423298	JQ423329	JQ423352	JQ423447	-
50	*T. fischeri*	Tsushima Is. 1, Nagasaki	34.65 N 129.44 E	5	ETOH	Mt65(4), Mt66	JQ423301, JQ423302	JQ423328	JQ423358	JQ423433, JQ423436	JQ423390, JQ423391, JQ423393
51	*T. fischeri*	Tsushima Is. 2, Nagasaki	34.38 N 129.33 E	5	ETOH	Mt65(4), Mt67	JQ423301, JQ423303	JQ423328	JQ423358	JQ423438	-
52	*T. fischeri*	Tsushima Is. 3, Nagasaki	34.12 N 129.17 E	3	ETOH	Mt68, Mt69, Mt70	JQ423298, JQ423304, JQ423305	JQ423328	JQ423352, 24	JQ423437	JQ423391, JQ423392
53	*T. fischeri*	Nagasaki, Nagasaki	32.58 N 129.78 E	4	ETOH	Mt46(3), Mt71	JQ423291	JQ423328	JQ423347, JQ423349	JQ423440, JQ423448	JQ423393
54	*T. fischeri*	Goto Is., Nagasaki	32.96 N 129.09 E	8	ETOH	Mt61(4) , Mt63, Mt72(3)	JQ423298, JQ423300	JQ423329	JQ423352, JQ423353	JQ423443	JQ423384
55	*T. fischeri*	Satsumasendai, Kagoshima	31.85 N 129.87 E	4	ETOH	Mt73(4)	JQ423292	JQ423328	JQ423347	JQ423441	JQ423389, JQ423396
56	*T. fischeri*	Jilin, China	42.01 N 126.40 E	2	ETOH	Mt74, Mt75	JQ423298	JQ423328	JQ423359, JQ423360	-	-
57	*T. fischeri*	Liaoning, China	41.31 N 123.66 E	2	ETOH	Mt74(2)	JQ423298	JQ423328	JQ423359	JQ423432	JQ423394
58	*T. fischeri*	Chungcheonbuk, South Korea	37.00 N 127.98 E	1	ETOH	Mt76	JQ423306	JQ423328	JQ423347	-	-
59	*T. fischeri*	Khabarovsk, Russia	50.53 N 137.09 E	4	ETOH	Mt77(4)	JQ423308	JQ423328	JQ423358	JQ423434	JQ423385, JQ423386
60	*T. fischeri*	Shebalino, Altai	53.36 N 83.56 E	3	ETOH	Mt78(3)	JQ423307	JQ423328	JQ423347	JQ423435	JQ423387, JQ423388
-	*T. kala*	Myanmar	-	1	Pinned	-	JQ423266	JQ423311	JQ423337	JQ423458	JQ423400
-	*T. filicaudis*	Taiwan	-	1	ETOH	-	JQ423265	JQ423310	JQ423336	JQ423457	-
-	*T. hainani*	Taiwan	-	1	ETOH	-	JQ423264	JQ423309	JQ423335	-	-

CHAPTER 3

Geometric morphometrics of wing venation reveals sexual dimorphism and intraspecific variation in wing size and shape of *Tongeia fischeri*

3.1 Abstract

Wing morphological variations are described here for the lycaenid *Tongeia fischeri*. A landmark-based geometric morphometric approach based on 22 landmarks of the forewing and 17 landmarks of the hindwing of 197 male and 187 female butterflies collected from 58 different localities in Japan was used to quantify wing size and shape variations between sexes and among genetically different populations. Sexual dimorphism in wing size and shape was detected. Females had significantly larger wings than males, while males showed a relatively elongated forewing with a longer apex and narrower wing tornus in comparison to females. Intraspecific variations in wing morphology among genetically different populations were revealed for the wing shape, but not wing size. Distinct wing shape differences were found in the vein intersections area around the distal part of the discal cell where median veins originated in the forewing and around the origin of the CU1 vein in the hindwing. This study is the first demonstrating the existence of wing morphological variation in *T. fischeri* and confirms morphological differentiation between populations that were previously classified by molecular markers. Furthermore, the results of this study also revealed consistency between phenetic trees inferred from wing shape variation and the phylogenetic tree obtained from molecular data, suggesting the presence of a phylogenetic signal in the wing shape of *T. fischeri*.

3.2 Introduction

The wing is an important organ for maintaining the fitness of an individual in winged insects (Pterygota). It plays an important role in many behavioral activities of insects, such as foraging, courtships, host-plant searching, migration, territoriality and predator avoidance (Betts & Wootton, 1988; Kingsolver & Koehl, 1994). Wing morphology is thus expected to be under strong selection and can reflect adaptation for these behavioral activities and/or adaptation to different environmental conditions by many modified forms of shape, size, structure, venation and pigmentation (Betts & Wootton, 1988; Bots *et al.*, 2012; Cockx, 2007; Hill *et al.*, 1999; Svensson & Friberg, 2007; Wootton, 1992). Morphological variation in insect wings is depended on environmental factors such as temperature (Bégin *et al.*, 2004),

host-plant (Soto *et al.*, 2008), altitude (Demirci *et al.*, 2012) and latitude (Outomuro & Johansson, 2011) as well as by internal genetic components (Birdsall *et al.*, 2000).

The insect wing consists of a cuticular membrane supported by a framework of veins, namely wing venation (Wootton, 1992). They serve both supporting the wing membrane and enclose circulating the transmission of hemolymph, oxygen and sometimes sensory information (Wootton, 1992).The wing venation pattern varies among orders of insects, with enormous variation in vein number, position and differentiation (De Celis & Diaz-Benjumea, 2003). Such variation is also observed between closely related species in the genus and even of between populations in the species (Albrecht & Kalia, 1997; Sotavolta, 1964).

Geometric morphometrics is a powerful approach that uses a set of landmark configurations to quantify biological shape, shape variation, and covariation of shape with other biotic or abiotic variables (Bookstein, 1996; Webster & Sheets, 2010; Zelditch *et al.*, 2004). Geometric morphometrics of wing venation has been used successfully to discriminate taxa in a complex species-group (Roggero & Passerin d'Entrèves, 2005), cryptic species (Muñoz-muñoz *et al.*, 2011; Schutze *et al.*, 2012), populations within a species (e.g. Kiyoshi & Hikida, 2012; Sadeghi *et al.*, 2009) and sexual dimorphism (Hernández-L. *et al.*, 2010). Several studies have used wing venation together with genetic data in evolutionary studies of divergent taxa/populations. Most of them revealed a high consistency between phenotypic and genetic variation and suggested a possible correlation between them (Miguel *et al.*, 2010; Muñoz-muñoz *et al.*, 2011; Schutze *et al.*, 2012).

Tongeia fischeri is a small butterfly in the family Lycaenidae. In Japan, it was recently divided into three subspecies, namely *T. f. japonica*, *T. f. shojii*, and *T. f. caudalis* (Satonaka, 2009; Shirôzu, 2006; Yago, 2007) or *T. f. shirozui* (Hida, 2005). These subspecies classifications are based mainly on the distinctions of morphological traits of the wing color and wing patterns. A recent phylogeographic study inferred from mitochondrial and nuclear sequences supported this classification, but suggested that the subspecies *T. f. caudalis* or *T. f. shirozui* should be noted as a variant forms of subspecies *T. f. fischeri* (Jeratthitikul *et al.*, 2013). The molecular study further revealed that *T. fischeri* in Japan consisted of six mitochondrial haplotype clades and one hybrid population between *T. f. shojii* and *T. f. fischeri* in northeastern Kyushu (Fig. 3-1). This hybrid population showed an evidence of mitochondrial introgression; the hybrids have mitochondrial DNA from *T. f. fischeri*, but nuclear DNA from *T. f. shojii* and *T. f. fischeri*. The authors argued that this phenomenon may have been caused by ancient hybridization followed by mitochondrial introgression of *T. f. fischeri* over *T. f. shojii*. In addition, a study of wing color pattern variation also noted that

the northeastern Kyushu population showed a wing pattern distinct from that of other *T. f. fischeri* in Kyushu, and instead more similar to *T. f. shoji* in Shikoku Island (Satonaka, 2003). However, hybrid forms of this population have apparently never been reported in any morphological study.

Although there is evidence of wing pattern variation and genetic variation between *T. fischeri* populations in Japan, a comprehensive study of morphological variation using reliable statistical approaches has not been conducted hitherto. In this study, geometric morphometric methods were used to quantify and analyze wing venation of *T. fischeri*. The first aim of this study was to determine wing size and shape variation between the sexes and among genetically different populations of *T. fischeri* in Japan. The variation detected in geometric analysis were then used to infer evolutionary relationships among the *T. fischeri* populations. The second aim was to evaluate the morphological similarity among these populations and to compare it with that of previously obtained from molecular studies (Jeratthitikul *et al.*, 2013).

3.3 Material and methods

3.3.1 Sample preparation and data acquisition

Tongeia fischeri specimens (197 male and 187 female butterflies, collected from 58 localities in Japan (Fig. 3-1)) were used in this study. Forewing and hindwing from each butterfly were prepared for making permanent slides by removing the covering scales, staining with 1% Eosin before mounting with EUKITT® mounting reagent on microscope slides. Usually the left wing was used; however, the right wing was used instead of the damaged left wing (for approximately 10% of the specimens, and evenly distributed across samples). Wing slide vouchers were deposited at The University Museum, The University of Tokyo, Japan.

Wing slides were randomly digitally photographed with a Leica EZ4D stereo microscope with the same camera setting. The nomenclature of wing venation followed the system proposed by Miller (1970). A set of 22 landmarks for the forewing and 17 landmarks for the hindwing covering the wing surface was selected (Fig. 3-2). We did not place the landmark at the location where the Sc vein meets the edge of the forewing because this location was not homologous across specimens. The wing images were randomly ordered in tpsUtil v.1.49 (Rohlf, 2012) before bi-dimensional coordination of landmarks was digitized using tpsDig2 v.2.16 (Rohlf, 2010).

3.3.2 Measurement error and repeatability

To ensure that the observed variation is attributable to biological variation and not to measurement error, all specimens were landmarked twice and Procrustes ANOVA (Klingenberg & McIntyre, 1998) was performed separately for each dataset in MORPHOJ v1.05c (Klingenberg, 2011). Repeatability (R) was calculated following the method of Arnqvist and Martensson (1998). R is the proportion of variance due to true variation among individuals relation to the total variance. It ranges between zero and one. Zero indicates that all variance is attributable to variance within individuals (100% measurement error). One indicates that all variance is found between individuals (0% measurement error). Landmark configurations for each specimen of two replications were then averaged using MORPHOJ, to reduce the possible effects of measurement error (Arnqvist & Martensson, 1998).

3.3.3 Data analyses

Centroid size (CS), the square root of the summed squared distance of each landmark from the centroids (Zelditch *et al.*, 2004), was calculated as an indicator of the overall size of the wing. Significant difference ($\alpha = 0.05$) of CS between populations and between sexes was assessed by an one-way analysis of variance (ANOVA) and Independent T-test, respectively, as implemented in PAST v2.17b (Hammer *et al.*, 2001).

For wing shape variation, averaged landmark data were first subjected to the Procrustes superimposition, which eliminates other variations (scale, position and orientation) except shape from samples (Dryden & Mardia, 1998). Wing size sometimes affects wing shape (i.e. allometry; Drake & Klingenberg, 2008). Multivariate regression of the wing-shape variable against centroid size as independent variable was thus conducted. The regression of the shape variable on centroid size of *T. fischeri* wings was statistically significant ($p < 0.0001$; 10,000 replicates of permutation tests), rejecting a null hypothesis of independence, and accounted for 2.58%, 1.83%, 4.91% and 3.13 % of shape variation for female forewing, male forewing, female hindwing and male hindwing, respectively. Accordingly, residual values corrected for allometric effect were used in further analyses. Samples were assigned into eight *a priori* populations characterized by unique genetic compositions detected in Jeratthitikul *et al.* (2013): (1) Central Honshu I and (2) Central Honshu II, (3) Western Honshu & Shikoku; (4) Kyushu I, (5) Kyushu II, (6) Ainoshima, (7) Tsushima, and (8) Hybrids — the hybrid population between *T .f. shojii* and *T. f. fischeri* (Fig.2-1). The Ainoshima population was assigned as a distinct group because of its different nuclear haplotype from other Kyushu populations (Jeratthitikul *et al.*, 2013). Canonical variate

analysis (CVA) was computed from covariance matrices in order to determine the relative difference in the wing shape among populations. Mahalanobis distances between pairwise populations were assessed for significant differences by the permutation test (10,000 iterations). The percentage of correct classification of a pair of populations was assessed by leave-one-out cross-validation as the implement of discriminant function analysis. All geometric morphometric analyses were carried out using MORPHOJ. This procedure was also applied to the samples in order to determine the wing-shape differences between males and females in each population.

In order to infer evolutionary relationships of wing-shape variation among *T.fischeri* populations, neighbour-joining trees (NJ; Saitou & Nei, 1987) based on Mahalanobis distances between population centroids derived from CVA were reconstructed using the web server T-REX. The Mahalanobis distances are listed in Tables 3-6 and 3-7.

3.4 Results

3.4.1 Measurement error and repeatability

Procrustes ANOVA indicated that the measurement error was negligible compared to the overall shape variation, as the mean squares for individual significantly exceeded the mean squares of the error, in all datasets ($p < 0.0001$; Table 3-1). Repeatability of the landmarks acquisition was high in all datasets (female forewing, R = 0.963; male forewing, R= 0.969; female hindwing, R = 0.966 and male hindwing, R = 0.967).

3.4.2 Sexual dimorphism

Females were significantly larger in forewing and hindwing centroid sizes than males in all populations (T-test: $p < 0.0001$; to 0.029; Fig. 3-3 and Table 3-2). The shape of forewing and hindwing were also different between males and females in all populations, (permutation test of Mahalanobis distances: $p < 0.001$; Table 3-3). In the forewing, males showed a relatively elongated wing (Fig. 3-4) with a longer apex (landmarks 15-17) and a narrower wing tornus (landmarks 20-22), in addition to the extension of landmarks 8-11 to the wing margin. In the hindwing, the shape differences were principally represented by landmarks 9 and 10 moving distally with respect to other landmarks in males, coupled with a slight proximal shift of landmarks 13-15 at the wing margin and landmarks 4 and 5 at the discal cell.

3.4.3 Wing size and shape variation between genetically different populations

Tongeia fischeri from eight populations did not differ significantly in wing centroid size in all datasets (ANOVA: p ranking from 0.072 to 0.801; Table 3-4), as indicated by the variations in the mean CS presented in Fig. 3-3.

In contrast, the shape analysis revealed shape variations among populations. The CVA revealed seven canonical variates, of which the first two CVs accounted for more than 60% of the variation (female forewing, 61.81%; male forewing, 64.88%; female hindwing, 64.80% and male hindwing, 64.02%; Table 3-5). *A priori* populations were significantly different from each other based on permutation tests for Mahalanobis distances for all comparisons and in all datasets ($p < 0.01$; Tables 3-6 and 3-7). Overall, individuals from the same population were clustered together and populations from the same subspecies were placed close to each other, with a considerable degree of overlap in some populations, especially between *T.f. shojii* (Western Honshu & Shikoku) and hybrid population (Fig. 3-5).

Wireframe modifications in Fig. 3-5 depicted the shape change from the consensus configuration along the two CV axes of each CVA plot. For the forewing of females (Fig.3-5A), individuals located in the positive portion of CV1 had elongated discal cell (landmarks 8-10), extended M1 and M2 veins (landmarks 17 and 18), and the crossvein between R1- Sc junction (landmark 3) and discal cell (landmark 4) was distally shifted when compared to the negative portion groups. Shape changes along CV2 were principally represented by a distal shift of landmark 11 as the score decreased, and the crossvein between the R1- Sc junction and discal cell was again distally shifted as the score increased. For the forewing of males (Fig. 3-5B), the elongation of the discal cell was more pronounced than that in females. The shape changes along CV2 again involved the distal shift of the crossvein between the R1- Sc junction and discal cell as the score decreased. In addition, individuals located in the positive portion of the scores had a broader termen (the distal region of the wing) when compared to the negative portion individuals (as described by landmarks 22).

Wing shape differences in the hindwings of both sexes showed a similar trend (Fig. 3-5C and D). Shape deformations along CV1were principally represented by the origin of the CU1 vein (landmarks 7) moving distally with respect to other landmarks as the score decreased in females and as the score increased in males, especially in Kyushu I population. In the hindwing of males, individuals placed in the positive portion of the CV1 axis had a narrower but broader wing (described by landmarks 11-13 and15) when compared to the negative portion individuals, who had a relatively elongated wing. This trend was also

observed, although it was less pronounced, in females. The shape changes along CV2 were largely indistinguishable, for example, the shifts of landmarks 9 and 12 in both sexes.

The percentage of correct classification of specimens according to cross-validation of DFA was 80.59% for female forewing; 87.33% for male forewing; 85.38% for female hindwing and 91.13% for male hindwing.

Unrooted neighbour-joining trees constructed from Mahalanobis distances between population centroids were similar for all datasets (Fig. 3-6A-D). They grouped *T.fischeri* populations into three groups, reflecting the subspecies classification. Hybrid populations were placed with Western Honshu and Shikoku populations; Central Honshu I and II were grouped together, whereas Kyushu I, Kyushu II, Tsushima and Ainoshima populations were more similar among themselves than they were with other subspecies.

3.5 Discussions

Sexual dimorphism in wing morphology (shape, size and color pattern) of butterflies and moths has long been known and appears to be driven largely by natural and sexual selection that affects males and females differently (Allen *et al.*, 2011). Our results showed that wings of females were significantly larger than those of males in all populations. As a proxy for body size (e.g. in Sullivan & Miller, 2007), larger wing size may indicate larger body size. This result confirms the trend of female-biased sexual dimorphism in size (females being larger than males), which is commonly found not only in Lepidoptera but also in other insects (Stillwell *et al.*, 2010). The selection for larger females was noted to be driven by natural selection for increased lifetime reproductive success, i.e. fecundity (Fischer & Fiedler, 2000; Reeve & Fairbairn, 1999). The patterns of selection on the small body size of males are not well understood but may be related to the period of their more rapid development during the larval stage relative to that of females, which is known as protandry (Fischer & Fiedler, 2000; Jarošík & Honek, 2007). The early eclosion of males may be favorable for sexual selection because the faster-developed males can increase the frequency and success of mating or increase the opportunity to access preferred territories, which would further enhance their fitness (Allen *et al.*, 2011; Morbey & Ydenberg, 2001). In addition, males butterflies may lose more weight during the transition from pupa to adult than females, suggesting that selective pressures may differ between the sexes (Fischer & Fiedler, 2001).

The results also showed a significant difference in wing shape between the sexes for *T. fischeri*. Sex differences in behavior related to flight are considered likely to exert selective pressures influencing the evolution of sexual dimorphism in wing shape (Allen *et al.*, 2011;

Gilchrist, 1990). Female butterflies tend to have patrolling flight in search of suitable foraging and host plants, while males spend most of their adult life locating sexually active females and guarding their territory. The short and broad wing characteristics observed in female *T. fischeri* would favor slow flight (Betts & Wootton, 1988). Scott (1983) suggested that females in several species of butterflies have wings designed for patrolling flight, including a less-pointed forewing and evenly convex margin. An elongated wing with narrowed tornus in male butterflies may indicate the capacity for extensive and highly agile flight (Betts & Wootton, 1988). This wing design presumably gives males high-powered flight for approaching a passing female or a rival male very quickly (Scott, 1983). Hernández-L. *et al.* (2010) also reported a similar pattern of elongated forewing in the male of *Tecia solanivora*.

The application of the geometric morphometric approach for genetically different populations of *T. fischeri* revealed that butterflies from eight populations did not differ significantly in the wing centroid size. In contrast, shape variations in the wings of *T. fischeri* were identified for both fore- and hindwing. The differences in wing shape, representes by wing venation, between genetic populations observed here are probably the result of population isolation according to the host-plant fragmentation, geographical isolation and climatic oscillations that occurred during Pleistocene (Jeratthitikul *et al.*, 2013). After isolation, local adaptation according to physical and biotic factors of a particular area could have been underlying ecological mechanisms that promoted morphological variation among the populations (Bots *et al.*, 2012; Cockx, 2007; Hernández-L. *et al.*, 2010; Hill *et al.*, 1999; Svensson & Friberg, 2007). Changes in the position of vein clusters can relate to the flight performance of butterflies. For instance, the forewing veins seem to be aggregated toward the costa in butterfly species in which males show perching behavior (Scott, 1983). In addition, considering the genetic basis of the formation of wing venation in insects (De Celis, 2003; De Celis & Diaz-Benjumea, 2003), it is possible that the phenotypic variation observed here may be influenced by the difference in genetic background of wing venation-regulating genes among populations.

Major morphometric differences among populations of *T. fischeri* were concerned with the wing vein intersections area around the distal part of the discal cell where median veins originated (landmarks 8-10) from the forewing and the origin of the CU1 vein (landmark 7) from the hindwing. It appears that the vein intersection inside the wing plays a more important role than the overall shape of the wing in population differentiation, suggesting that the latter character may be more conserved across the populations than the

former character. However, both of these characters contributed to explaining the variation in the male hindwing data set. This suggests that the degree of variation in wing shape depends on the organ examined as well as the sex.

The wing shape of the hybrid population was more similar to that of Western Honshu and Shikoku than to those of other populations, as revealed by morphospace plots in Fig. 3-5 and phenetic trees in Fig. 3-6. This result is consistent with a previous report on wing color pattern. The populations in northeastern Kyushu have been noted for a wing color pattern distinct from those of other *T. f. fischeri* in Kyushu, and more similar to that of *T. f. shojii* in Shikoku Island. They were thus classified as a population of subspecies *T. f. shojii* (Satonaka, 2003). However, despite the fact that hybridization in this population was supported by molecular data (Jeratthitikul *et al.*, 2013), an intermediate form of this population was not observed in the present study.

3.6 Conclusions

In conclusion, the present study revealed morphological variations in the wings, namely in their size and shape, associated with sex in *T. fischeri*. Furthermore, variations in the wing shape, but not the wing size, among genetically different populations were revealed. This study is the first showing the existence of wing morphological variation in *T. fischeri*, and confirms the morphological differentiation among populations previously classified by molecular analysis. The results also revealed consistency between the evolutionary relationships inferred from wing shape variation and the phylogenetic relationship obtained from molecular data, suggesting the presence of a phylogenetic signal in the wing shape of *T. fischeri*. As proven in this study and some other studies (Hernández-L. *et al.*, 2010; Roggero & Passerin d'Entrèves, 2005), wing venation varies greatly among geographically/ genetically diverged populations and can be used as a diagnostic character to recognize taxa or evolutionary lineages and as an evolutionary character in further systematic or evolutionary studies.

Figure legends

Figure 3-1 Map of the geographical distribution of *Tongeia fischeri* in Japan showing the subspecies boundaries and locations from which specimens were collected. The symbols denote *a priori* population groups used in this study.

Figure 3-2 Forewing (upper) and hindwing (lower) of *Tongeia fischeri* showing wing venation nomenclature and landmarks (black circles) used in this study. SC, subcosta; R1, R2, R3, R4+5, radius and branches; M1, M2, medius and branches; Cu1, Cu2, cubitus and branches; A1+2, anal; Sc+R1, subcosta and the fist radius; Rs, radial sector.

Figure 3-3 Box-and-whisker plots showing variation in forewing (A) and hindwing (B) centroid size in populations and sexes of *Tongeia fischeri* in Japan. Boxes show the median, 25th and 75th percentiles, whiskers show minimum and maximum observations, and circles show outliers.

Figure 3-4 Wing shape differences between sexes of *Tongeia fischeri* represented by wireframes for females (solid lines) and males (dotted lines).

Figure 3-5 Plots of individual scores for the first two canonical variates (CV1 and CV2) derived from canonical variate analysis (CVA) for the total variation in the wing venation of *Tongeia fischeri*. Wireframes showing the shape changes (solid lines) from the consensus configuration of landmarks (dotted lines) to each extreme negative and positive CV score. (A) female forewing, (B) male forewing, (C) female hindwing and (D) male hindwing. Symbol explanations are indicated in Fig. 3-1.

Figure 3-6 Relationships among *Tongeia fischeri* populations. (A-D) Unrooted neighbor-joining trees based on Mahalanobis distances between population centroids derived from the canonical variates analysis. (A) female forewing, (B) male forewing, (C) female hindwing and (D) male hindwing. (E) Phylogenetic tree based on combined dataset of mitochondrial and nuclear DNA taken from Jeratthitikul *et al.* (2013) with modification: bipartition posterior probability of Bayesian inference (BI) and bootstrap value for Maximum Likelihood (ML) analyses are indicated (ML/BI).

Figure 3-1

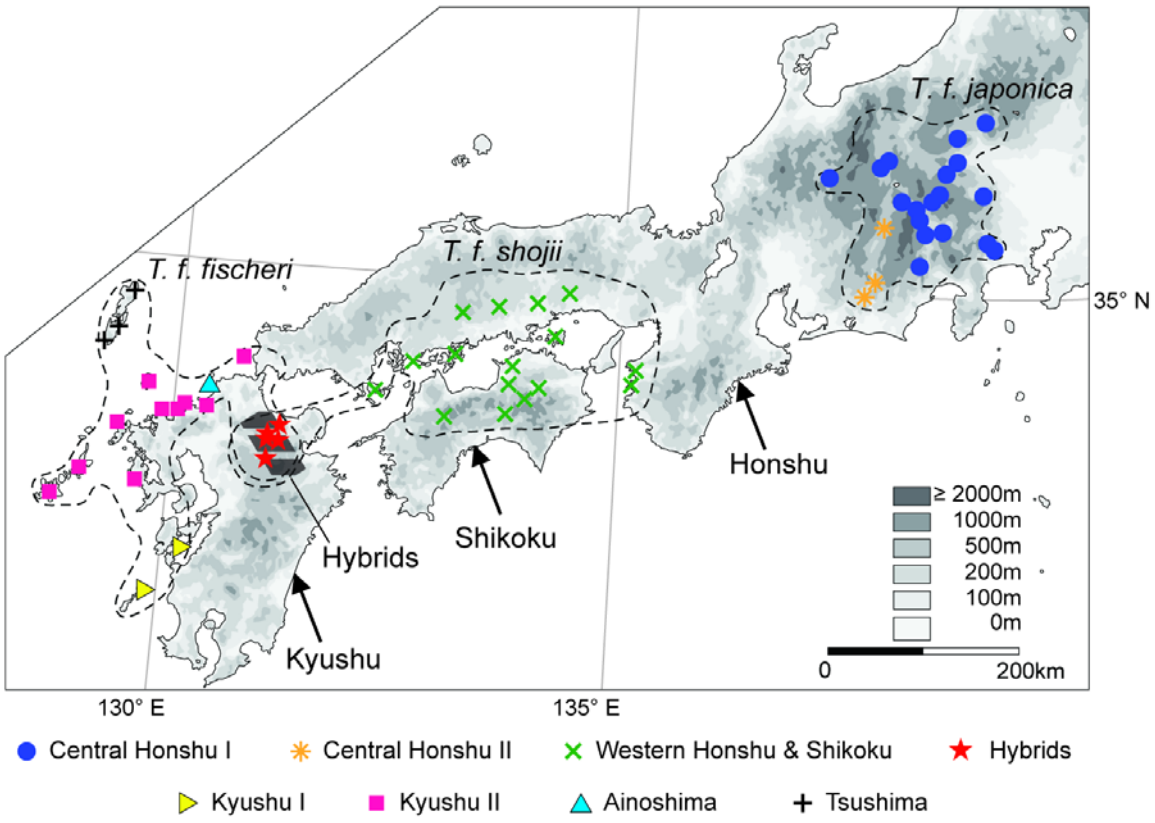

T. f. japonica
T. f. shojii
T. f. fischeri
35° N
Honshu
Shikoku
Hybrids
Kyushu
≥ 2000m
1000m
500m
200m
100m
0m
0
200km
130° E
135° E
Central Honshu I
Central Honshu II
Western Honshu & Shikoku
Hybrids
Kyushu I
Kyushu II
Ainoshima
Tsushima

Figure 3-2

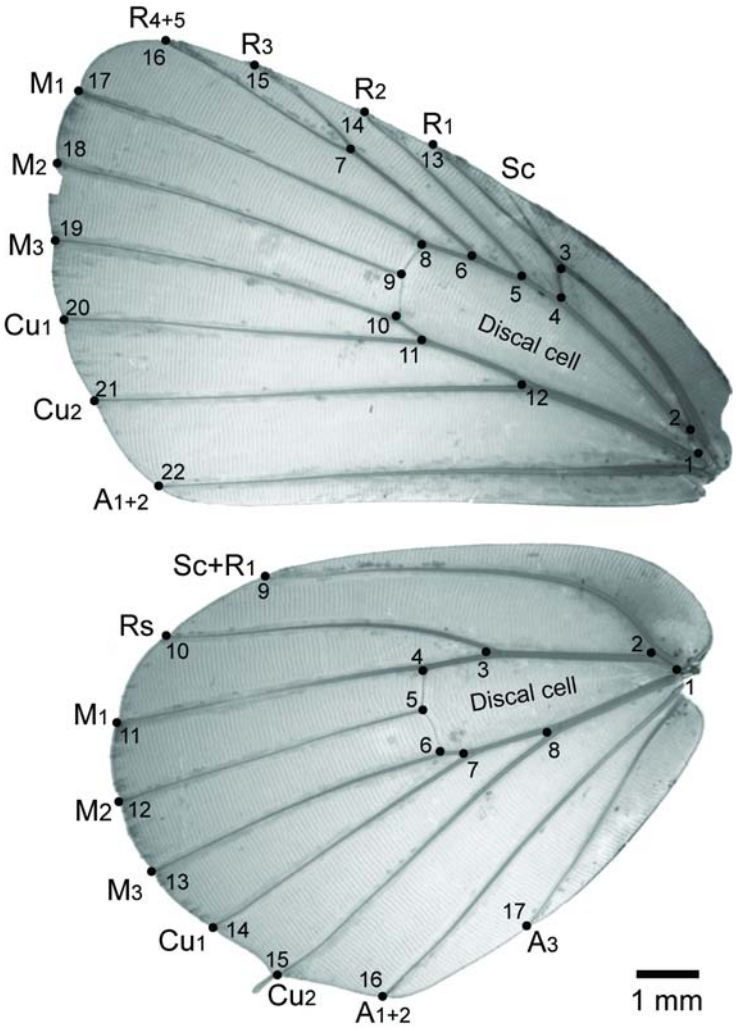

R4+5
R3
R2
R1
Sc
M1
M2
M3
Cu1
Cu2
A1+2
Discal cell
Sc+R1
Rs
M1
M2
M3
Cu1
Cu2
A1+2
A3
Discal cell
1 mm

Figure 3-3

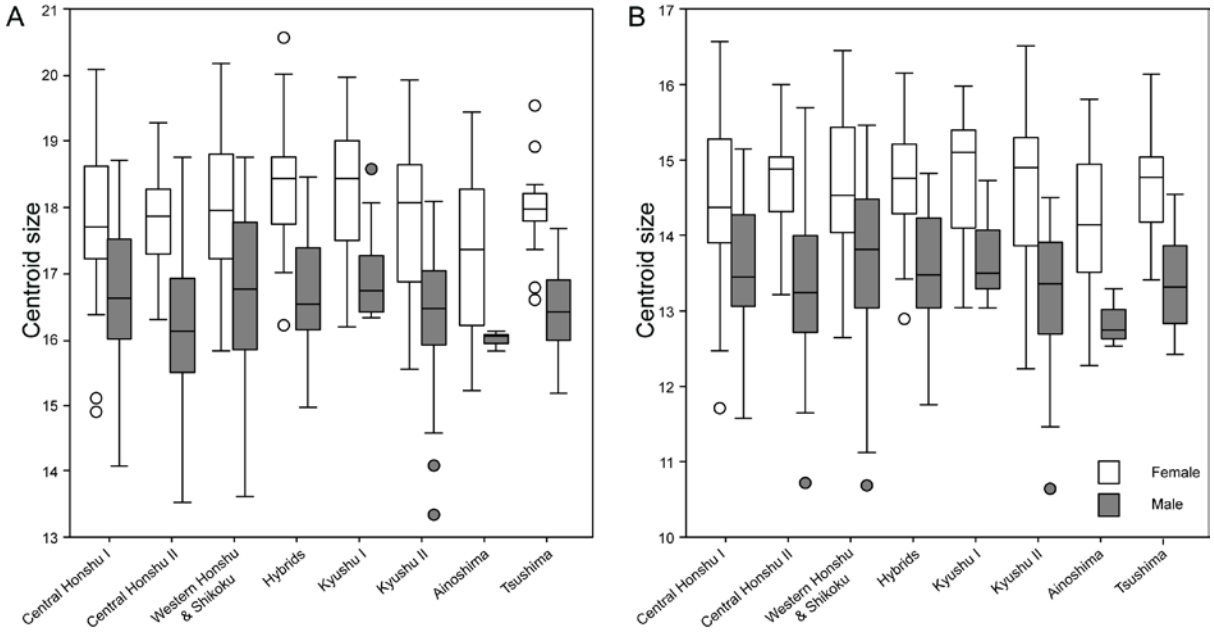

A
B
Centroid size
Female
Male
Central Honshu I
Central Honshu II
Western Honshu & Shikoku
Hybrids
Kyushu I
Kyushu II
Ainoshima
Tsushima

Figure 3-4

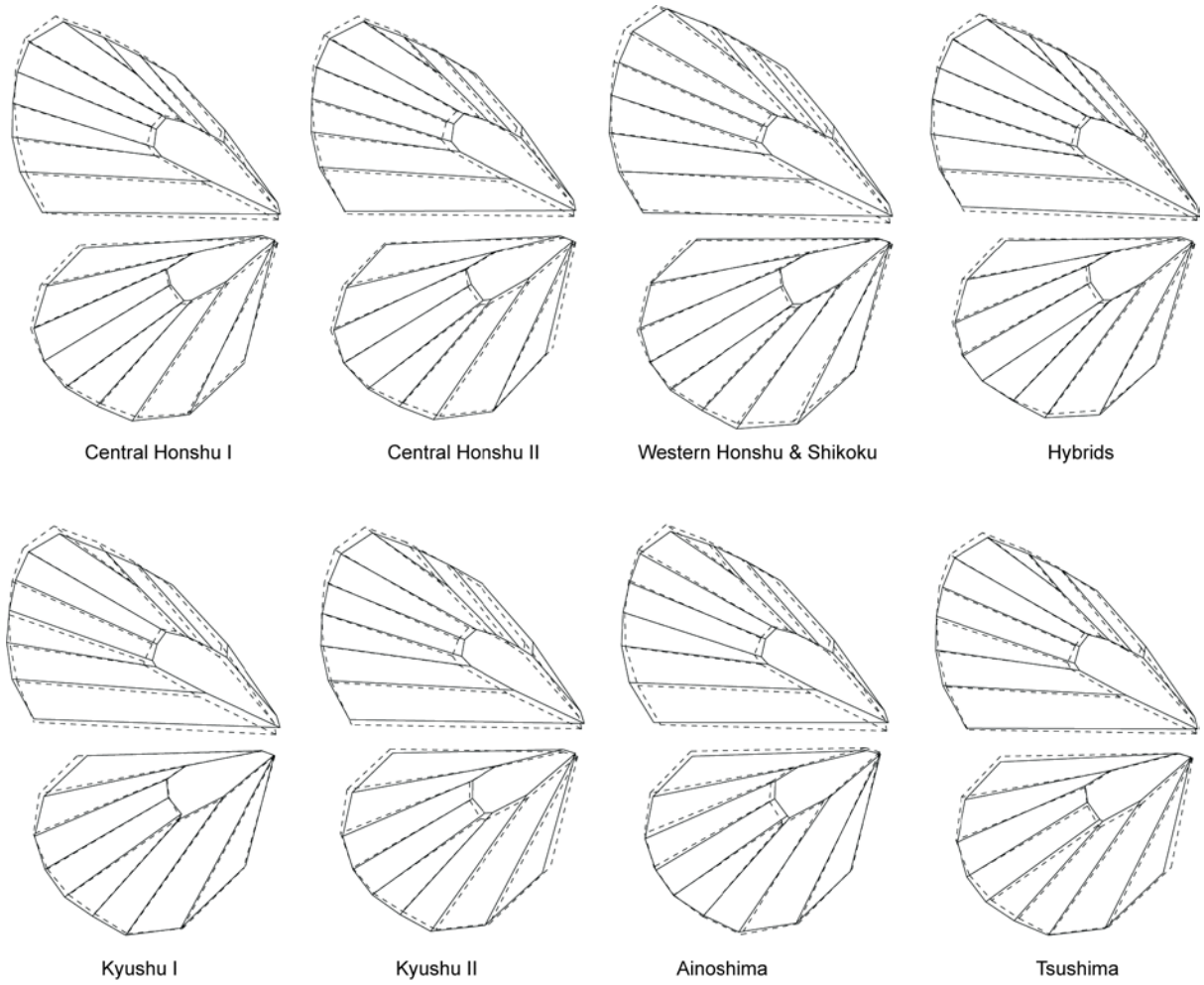

Central Honshu I
Central Honshu II
Western Honshu & Shikoku
Hybrids
Kyushu I
Kyushu II
Ainoshima
Tsushima

Figure 3-5

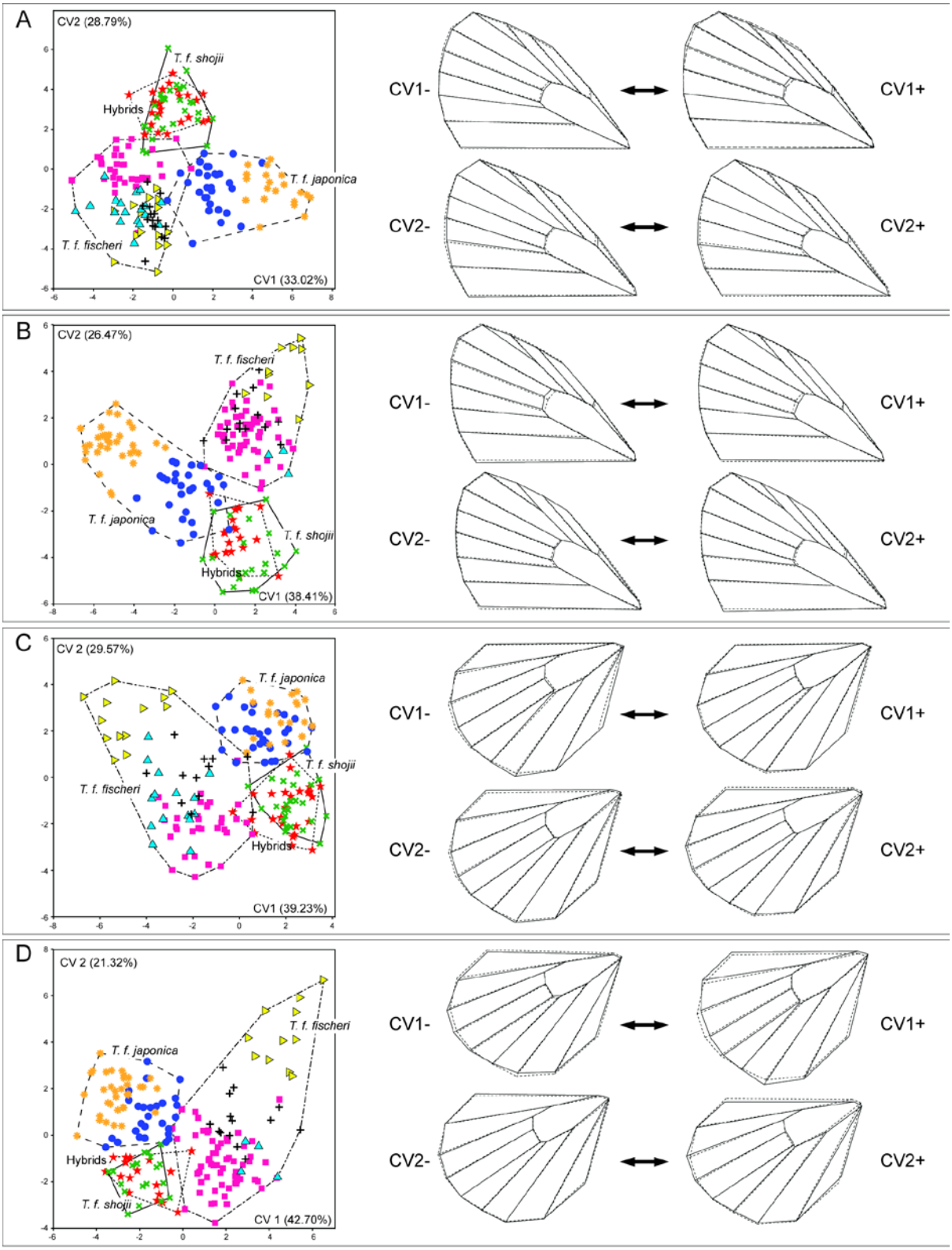

A
CV2 (28.79%)
T. f. shojii
Hybrids
T. f. japonica
T. f. fischeri
CV1 (33.02%)
CV1-
CV1+
CV2-
CV2+
B
CV2 (26.47%)
T. f. fischeri
T. f. japonica
T. f. shojii
Hybrids
CV1 (38.41%)
CV1-
CV1+
CV2-
CV2+
C
CV 2 (29.57%)
T. f. japonica
T. f. shojii
T. f. fischeri
Hybrids
CV1 (39.23%)
CV1-
CV1+
CV2-
CV2+
D
CV 2 (21.32%)
T. f. fischeri
T. f. japonica
Hybrids
T. f. shojii
CV 1 (42.70%)
CV1-
CV1+
CV2-
CV2+

Figure 3-6

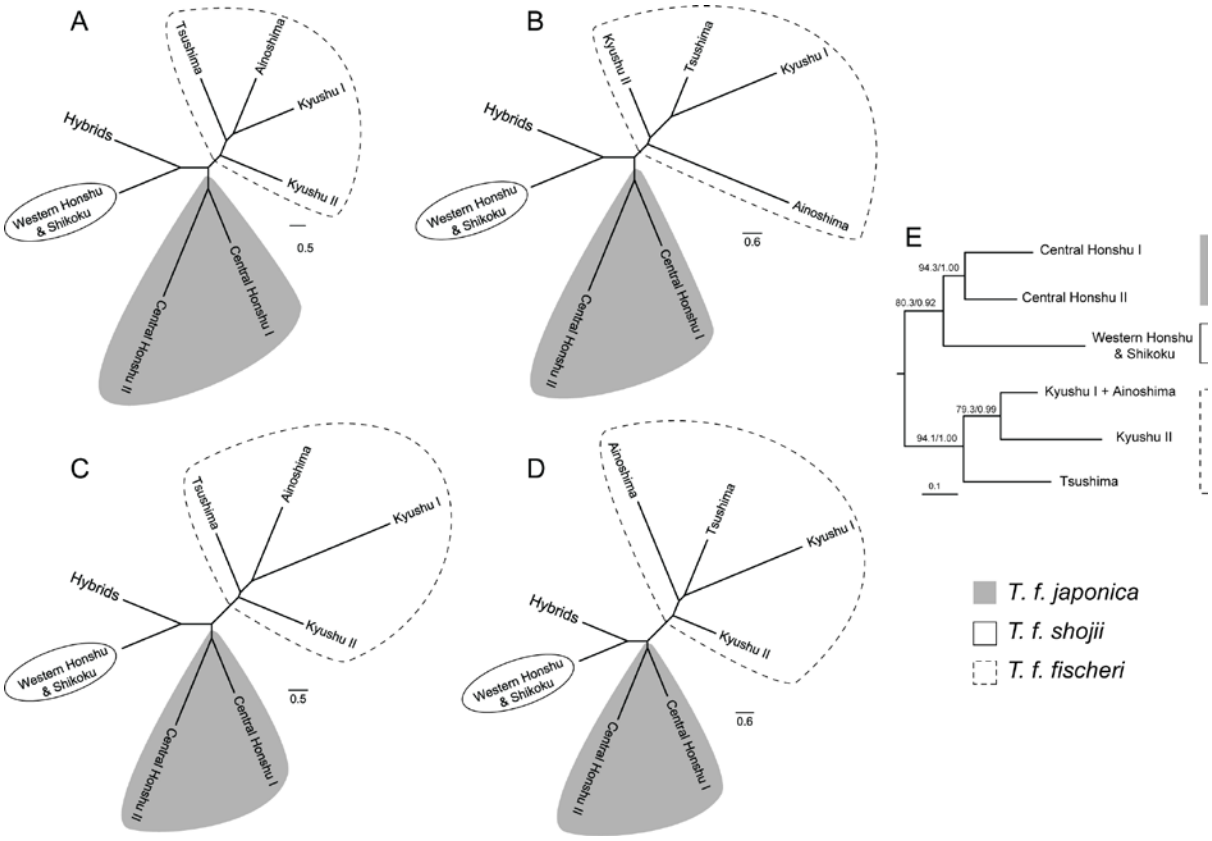

A
Tsushima
Ainoshima
Kyushu I
Hybrids
Kyushu II
Western Honshu & Shikoku
Central Honshu I
Central Honshu II
0.5
B
Kyushu II
Tsushima
Kyushu I
Hybrids
Ainoshima
Western Honshu & Shikoku
Central Honshu I
Central Honshu II
0.6
C
Tsushima
Ainoshima
Kyushu I
Hybrids
Kyushu II
Western Honshu & Shikoku
Central Honshu I
Central Honshu II
0.5
D
Ainoshima
Tsushima
Kyushu I
Hybrids
Kyushu II
Western Honshu & Shikoku
Central Honshu I
Central Honshu II
0.6
E
Central Honshu I
94.3/1.00
Central Honshu II
80.3/0.92
Western Honshu & Shikoku
Kyushu I + Ainoshima
79.3/0.99
Kyushu II
94.1/1.00
Tsushima
0.1
T. f. japonica
T. f. shojii
T. f. fischeri

Table 3-1 Procrustes ANOVA (Klingenberg & McIntyre, 1998) for analysis of measurement error in landmark acquisition. Individual represents overall variation among samples in the dataset. Error is the measurement error calculated from the variation among repeated measurements of the same sample. SS, sum of squares; MS, mean squares; df, degrees of freedom; *F*, *F* statistic and *p*, statistical significance.

	Source of variation	**SS**	**MS**	**df**	***F***	***p***
Female forewing	Individual	0.51655389	7.05675E-05	7320	52.89	<0.0001
	Error	0.0098207	1.3343E-06	7360		
Male forewing	Individual	0.5626086	7.60282E-05	7400	63.01	<0.0001
	Error	0.0089775	1.2067E-06	7440		
Female hindwing	Individual	0.6295992	0.000119924	5250	57.09	<0.0001
	Error	0.0110906	2.1005E-06	5280		
Male hindwing	Individual	0.7214657	0.000129994	5550	60.08	<0.0001
	Error	0.0120724	2.1635E-06	5580		

Table 3-2 Independent T-test of forewing (left panel) and hindwing centroid size (right panel) between females and males with mean of centroid sizes in eight populations of *Tongeia fischeri*. SD, standard deviation; *t*, t-score; df, degrees of freedom and *p*, statistical significance.

Populations	Forewing					Hindwing				
	t	df	*p*	Mean (±SD)		*t*	df	*p*	Mean (±SD)	
				Female	Male				Female	Male
Central Honshu I	3.463	55	0.001	17.81(1.22)	16.76(1.05)	3.42	55	0.001	14.53(1.15)	13.60(0.86)
Central Honshu II	6.257	52	<0.0001	17.83(0.76)	16.09(1.10)	5.846	53	<0.0001	14.67(0.70)	13.24(0.98)
Western Honshu & Shikoku	3.639	46	0.001	18.06(1.21)	16.66(1.44)	2.861	44	0.006	14.68(1.07)	13.66(1.34)
Hybrids	5.753	43	<0.0001	18.33(0.94)	16.71(0.93)	4.453	40	<0.0001	14.61(0.79)	13.49(0.80)
Kyushu I	3.009	21	0.007	18.26(1.07)	17.05(0.77)	3.418	22	0.002	14.76(0.86)	13.71(0.59)
Kyushu II	7.274	88	<0.0001	17.92(1.07)	16.37(0.92)	7.285	91	<0.0001	14.64(0.95)	13.26(0.84)
Ainoshima	4.423	20.994	<0.001	17.34(1.28)	16.01(0.16)	2.39	17	0.029	14.19(1.10)	12.84(0.32)
Tsushima	5.889	28	<0.0001	17.95(0.73)	16.44(0.68)	4.579	24	<0.001	14.69(0.82)	13.38(0.65)

Table 3-3 Eigenvalues and Mahalanobis distances derived from canonical variate analysis (CVA) for wing venation shape between females and males of *Tongeia fischeri*. *P-values* of Mahalanobis distances were calculated by 10,000 random permutations per test to determine statistical significance of differences between sexes.

	Populations	**Eigenvalues**	**Mahalanobis distances (*p-values*)**
Forewing			
	Central Honshu I	23.25	9.4739 (< 0.0001)
	Central Honshu II	26.73	10.5065 (< 0.0001)
	Western Honshu & Shikoku	51.50	14.1616 (< 0.0001)
	Hybrids	49.34	13.9015 (< 0.0001)
	Kyushu I	10.95	6.3773 (< 0.0001)
	Kyushu II	11.52	6.9224 (< 0.0001)
	Ainoshima	45.29	7.4504 (0.0001)
	Tsushima	6.90	13.0025 (< 0.0001)
Hindwing			
	Central Honshu I	3.57	3.7172 (<0.0001)
	Central Honshu II	5.22	4.6170 (<0.0001)
	Western Honshu & Shikoku	7.56	5.4629 (<0.0001)
	Hybrids	7.91	5.5927 (<0.0001)
	Kyushu I	23.31	9.2771 (<0.0001)
	Kyushu II	1.98	2.908 (<0.0001)
	Ainoshima	18.01	8.1788 (<0.0001)
	Tsushima	8.65	6.8222 (0.0002)

Table 3-4 One-way ANOVA on forewing (left panel) and hindwing (right panel) centroid size in eight populations of *Tongeia fischeri*. SS, sum of squares; MS, mean squares; df, degrees of freedom; *F*, *F* statistic and *p*, statistical significance.

	Source of variation	Forewing					Hindwing				
		SS	**df**	**MS**	***F***	***p***	**SS**	**df**	**MS**	***F***	***p***
Female											
	Between Groups	13.6953	7	1.9565	1.6969	0.112	3.5128	7	0.5018	0.5433	0.801
	Within Groups	202.9215	176	1.1530			155.1720	168	0.9236		
	Total	216.6168	183				158.6847	175			
Male											
	Between Groups	13.7861	7	1.9694	1.8973	0.072	6.9027	7	0.9861	1.2203	0.294
	Within Groups	184.7712	178	1.0380			143.8328	178	0.8080		
	Total	198.5574	185				150.7355	185			

.

Table 3-5 Eigenvalues and percentages of variance explained by seven canonical variates (CVs) produced from canonical variate analysis (CVA) of the wing venation of *Tongeia fischeri*.

CV	Forewing				Hindwing			
	female		male		female		male	
	Eigenvalues	% Variance	Eigenvalues	% Variance	Eigenvalues	% Variance	Eigenvalues	% Variance
1	5.41	33.02	7.40	38.41	5.28	39.23	6.10	42.70
2	4.71	28.79	5.10	26.47	3.44	25.57	3.04	21.32
3	1.91	11.69	2.43	12.63	1.55	11.51	2.02	14.13
4	1.53	9.35	1.99	10.35	1.25	9.28	1.29	9.04
5	1.28	7.83	1.17	6.10	0.92	6.82	0.80	5.57
6	0.89	5.46	0.69	3.56	0.55	4.11	0.65	4.52
7	0.63	3.86	0.48	2.50	0.47	3.48	0.39	2.73

Table 3-6 Mahalanobis distances of *Tongeia fischeri* populations derived from canonical variate analysis (CVA) of the forewing with *p-values* calculated by 10,000 random permutations per test to determine statistical significance of differences between pairs of populations. Above diagonal is female; below diagonal is male.

Populations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. Central Honshu I	-	5.6244 $p < 0.0001$	5.6085 $p < 0.0001$	6.0411 $p < 0.0001$	5.8935 $p < 0.0001$	5.5492 $p < 0.0001$	5.8408 $p < 0.0001$	5.2705 $p < 0.0001$
2. Central Honshu II	6.1462 $p < 0.0001$	-	7.2602 $p < 0.0001$	7.4489 $p < 0.0001$	7.4681 $p < 0.0001$	7.9054 $p < 0.0001$	8.0276 $p < 0.0001$	7.3448 $p < 0.0001$
3. Western Honshu & Shikoku	6.3111 $p < 0.0001$	8.5033 $p < 0.0001$	-	4.4342 $p < 0.0001$	6.9207 $p < 0.0001$	5.0851 $p < 0.0001$	6.3505 $p < 0.0001$	6.5596 $p < 0.0001$
4. Hybrids	6.2609 $p < 0.0001$	7.7789 $p < 0.0001$	4.8021 $p < 0.0001$	-	6.7645 $p < 0.0001$	5.4406 $p < 0.0001$	6.3883 $p < 0.0001$	6.7055 $p < 0.0001$
5. Kyushu I	8.7736 $p < 0.0001$	9.8407 $p < 0.0001$	9.0170 $p < 0.0001$	9.0346 $p < 0.0001$	-	5.2973 $p < 0.0001$	4.0205 $p < 0.0001$	4.3680 $p < 0.0001$
6. Kyushu II	5.5186 $p < 0.0001$	6.9787 $p < 0.0001$	6.2400 $p < 0.0001$	5.6380 $p < 0.0001$	6.4521 $p < 0.0001$	-	4.4753 $p < 0.0001$	5.0012 $p < 0.0001$
7. Ainoshima	8.4090 $p < 0.0001$	10.7382 $p = 0.0002$	8.4702 $p = 0.0002$	8.9375 $p = 0.0006$	9.2401 $p = 0.0032$	7.1147 $p < 0.0001$	-	4.5788 $p < 0.0001$
8. Tsushima	6.3347 $p < 0.0001$	7.2199 $p < 0.0001$	6.8677 $p < 0.0001$	6.3930 $p < 0.0001$	5.0454 $p < 0.0001$	3.9166 $p < 0.0001$	7.8107 $p = 0.001$	-

Table 3-7 Mahalanobis distances of *Tongeia fischeri* populations derived from canonical variate analysis (CVA) of the hindwing with *p-values* calculated by 10,000 random permutations per test to determine statistical significance of differences between pairs of populations. Above diagonal is female; below diagonal is male.

Populations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. Central Honshu I	-	4.0402 $p < 0.0001$	4.6334 $p < 0.0001$	4.2618 $p < 0.0001$	6.9306 $p < 0.0001$	5.2823 $p < 0.0001$	5.7705 $p < 0.0001$	4.5034 $p < 0.0001$
2. Central Honshu II	4.4212 $p < 0.0001$	-	5.0157 $p < 0.0001$	5.5642 $p < 0.0001$	7.8311 $p < 0.0001$	6.0964 $p < 0.0001$	6.8035 $p < 0.0001$	5.6346 $p < 0.0001$
3. Western Honshu & Shikoku	4.7226 $p < 0.0001$	5.1922 $p < 0.0001$	-	3.3356 $p < 0.0001$	8.3841 $p < 0.0001$	4.9540 $p < 0.0001$	6.0338 $p < 0.0001$	5.5542 $p < 0.0001$
4. Hybrids	4.0422 $p < 0.0001$	5.4214 $p < 0.0001$	3.4450 $p < 0.0001$	-	8.3018 $p < 0.0001$	4.8293 $p < 0.0001$	6.2529 $p < 0.0001$	5.5058 $p < 0.0001$
5. Kyushu I	7.8330 $p < 0.0001$	9.0805 $p < 0.0001$	9.1872 $p < 0.0001$	9.1717 $p < 0.0001$	-	6.7637 $p < 0.0001$	6.0485 $p < 0.0001$	6.0133 $p < 0.0001$
6. Kyushu II	4.9086 $p < 0.0001$	5.8735 $p < 0.0001$	5.0899 $p < 0.0001$	5.0846 $p < 0.0001$	7.1302 $p < 0.0001$	-	4.0073 $p < 0.0001$	3.7822 $p < 0.0001$
7. Ainoshima	7.8640 $p < 0.0001$	8.5571 $p < 0.0001$	7.7885 $p < 0.0001$	7.5163 $p = 0.0002$	7.9371 $p = 0.0006$	5.5613 $p < 0.0001$	-	4.2388 $p < 0.0001$
8. Tsushima	6.2210 $p < 0.0001$	6.7068 $p < 0.0001$	6.4348 $p < 0.0001$	6.4840 $p < 0.0001$	6.4223 $p < 0.0001$	4.2816 $p < 0.0001$	6.3527 $p < 0.0001$	-

CHAPTER 4

General discussion and conclusion

4.1 Colonization routes of Japanese terrestrial animals

It is believed that the connection between Japan and the continent has occurred at least two locations during the formation of the islands. The first connection was since the early formation. Western Japan was connected to eastern China and Korea Peninsula as a land bridge about 10 to 1.7 million years ago (Mya). This connection was separated latter by Tsushima strait around 3.9 to 0.8 Mya and connected again during the last glacial maximum (Barnes, 2003; Kaizuka, 1980; Kitamura & Kimoto, 2004; 2006; Yonekura *et al.*, 2001). The second connection was at the northern islands (Hokkaido at present), which was temporarily connected to the continent and Japanese mainland during the glacial periods of the late Pleistocene (Kaizuka, 1980; Yonekura *et al.*, 2001). Present Japanese insects may have colonized Japan using these two land connections(Fig. 4-1), in addition to some species that existed in Japan since the Pliocene and early Pleistocene (Sota & Hayashi, 2007).

The phylogeographic study in Chapter 2 suggests that ancestors of *T.fischeri* might have colonized Japan using a land bridge or narrow channel between western Japan and the Korean Peninsula and passing Tsushima Islands to Kyushu and Japanese mainland. This hypothesis is supported by the haplotype sharing between southern Tsushima and Kyushu populations in mitochondrial dataset. Haplotype Mt70 is positioned near the root of the K-II clade of mitochondrial network (Fig. 2-3d), and can be considered as the most ancestral allele in the K-II clade. This route is belived to have contributed greatly to the present fauna in the main islands (Sota & Hayashi, 2007). Other Japanese insects that use this migration route are, for examples, the migratory locust, *Locusta migratoria* (Tokuda *et al.*, 2010) and the carabid ground beetles (Tominaga *et al.*, 2000).

Continental ancestors of *T. fishceri* may have also migrated to the most northeastern part of the Asian continent, evidenced by a distribution of one subspecies, namely *T. f. sachalinensis*, in Sakhalin Island (Yakovlev, 2003). However, the subspecies has not migrated southward to Japan.

The timing of major continental dispersal event of *T. fischeri* to Japan may have been around 0.29 Mya in the mid-Pleistocene, as suggested by divergence time analysis in Chapter 2. This finding is consistent with other studies that used comprehensive information from fossil records. They proposed that colonization events of Japanese insects may occurred

during the mid-Pleistocene (Sota & Hayashi, 2007). However, this thesis used a universal arthropod mtDNA substitution rate of 2.3% divergence per million years (Brower, 1994) for the tree dating. The use of substitution rates taken from literature should be applied with caution because mitochondrial substitution rate usually varies across taxa and animal groups (Papadopoulou *et al.*, 2010 and reference therein). The previous molecular phylogenetic analyses of Japanese insects based on molecular clocks taken from other studies and with out robust calibrations (e.g. geological events or fossil records) suggested that there are vast variations in the timing of colonization from the continental ancestors, for examples, Miocene in the carabid ground beetles (Tominaga *et al.*, 2000), Pliocene in the dragonfly genus *Davidius* (Kiyoshi & Sota, 2006), and mid- to late Pleistocene in the coastal tiger beetles (Satoh *et al.*, 2004).

4.2 Utility of using multilocus markers in phylogeographic study

The marker of choice for evolutionary history inference since the development of phylogeography has been the mitochondrial DNA. It possess many advantages over other molecular markers and gains popularity for animal phylogeographic study for a longtime (Avise, 2000; Freeland, 2005). However, recently, phylogeography has been shifted toward the use of multilocus data of mitochondrial and nuclear DNA markers due to advancement of nuclear sequences acquisition (Brito & Edwards, 2009). Nuclear genomic data for phylogeographic studies can be obtained by several approaches in addition to direct sequencing, including next-generation sequencing (NGS; McCormack *et al.*, 2013), single nucleotide polymorphisms (SNP; Brito & Edwards, 2009), amplified fragment length polymorphisms (AFLP; Creer *et al.*, 2004), microsatellites (Zink & Barrowclough, 2008), and allozyme (Chaturvedi *et al.*, 2011).

This thesis has shown an advantage of multilocus markers in phylogeographic study (Chapter 2). If only mitochondrial genes were used, phylogeographic pattern of *T. fischeri* would solely conclude that Kyushu population was separated from Honshu population and fail to reveal the ancient hybridization followed by mitochondrial introgression in northeastern Kyushu populations. Apart from the success in detecting mitochondrial introgression of *T. fischeri* populations in this study, usage of combined data between mitochondrial and nuclear genes has been proven effective in many phylogeographic studies. For example, recent research using multilocus data has revealed multiple expansion in demographic history (Eytan & Hellberg, 2010); cryptic species (Wielstra *et al.*, 2013); introgression causes genealogical discordance in host races (Ohshima & Yoshizawa, 2010)

and male-biased dispersal pattern (Dai *et al.*, 2013). The finding of this study and other literatures suggest that either mitochondrial or nuclear markers alone would not have provided a complete picture of the historical demography and phylogeographic pattern, but the use of multilocus allows researchers to answer more complicated evolutionary questions of target organisms (Hare, 2001; Zink & Barrowclough, 2008). However, there are at least two practical concerns regarding the use of nuclear DNA genes for phylogeography. First, phylogenetic method of gene tree should take into consideration the concern of recombination. Second, low mutation rates of nuclear gene may result in poor resolution phylogenetic trees due to too few informative polymorphisms (Hare, 2001).

Nevertheless, an application of nuclear DNA sequences in phylogeographic study of Japanese animal is relatively rare. This may be due to difficulties in obtaining nuclear DNA sequences. As nuclear DNA usually occurrs in animal cell as a single copy, the amplification is difficult, especially when dealing with poor quality DNA of long-term storage samples or dry museum specimens. In addition, primer sets for nuclear genes are still limited. Some primers that work in some species may fail in other species. Moreover, nuclear gene sometime occurred in animal cell as heterozygous allele, which usually cannot be read with a direct sequencing. To separate this heterozygous allele, sub-cloning using vector and bacterial component cell is needed. This approach is expensive and can increase budget of the study.

4.3 Hybrid zone in northeastern Kyushu

One important result in this study was the finding of hybrid zone with mitochondrial introgression between two populations/subspecies of *T. fischeri*. Mitochondrial haplotypes from the Kyushu population are suspected to have been introduced and become fixed over the expanding Shikoku population in northeastern Kyushu where these two populations have hybridized. Nuclear DNA alleles of both parent populations can be found in this area. Two specimens from Oita (locality 46, Fig. 2-1) showed an intermediate genotype (hybrids), containing haplotypes from both Kyushu and Shikoku populations (Chapter 2). However, although this hybrid zone was strongly supported by molecular data, an intermediate morphological form was not observed in the wing venation. The wing shape of northeastern Kyushu population was more similar to Shikoku populations than other butterflies of Kyushu populations. Only few individuals shared wing shape with Kyushu populations (Chapter 3).

Hybrid zone can be defined as narrow regions where genetically distinct populations meet, mate, and produce hybrids (Barton & Hewitt, 1985). Hybrid zone may be roughly

classified into two types depending on the distribution of genotypic classes (Harrison & Bogdanowicz, 1997). One is ‘unimodal’ hybrid zone in which intermediate hybrid genotypes are common. The other is ‘bimodal’ hybrid zone in which populations predominantly consist of individuals genetically similar to one or another parental genotype and intermediate forms are rare (Harrison & Bogdanowicz, 1997). The latter case seems to be rarer and typically associated strongly with prezygotic isolation, which often lead to assortative mating within the hybrid populations (Jiggins & Mallet, 2000). The hybrid zone discovered in this study seems to fit with a bimodal hybrid zone. Most specimens from northeastern Kyushu consist of nuclear DNA haplotypes from parental populations in both Rpl5 and Ldh nuclear genes. Only two out of 18 individuals shown intermediate forms contained one allele from Kyushu and another from Shikoku population. The allele frequency of two parental alleles in the population varies among two nuclear gene examined here. There is a higher frequency of Shikoku alleles of Ldh gene in this population, whereas more Kyushu genotypes of Rpl5 gene are found in the population. This suggests that an individual's nuclear genome may have strikingly different evolutionary histories resulting from different type of selection or different genetic drift process.

This hypothesis is likely an explanation for the wing shape, which is directly affected by genetically components. The Shikoku phenotype may favor for this region rather than the Kyushu phenotype and has been selected by the external environment. Considering the microhabitat of *T. fischeri*, Kyushu populations are mainly distributed in coastal area where strong wind is common, while Shikoku populations are living in inland habitat that usually has no strong wind. Microhabitat in northeastern Kyushu is similar to the habitat in Shikoku. Therefore, the wing type of inland habitat of Shikoku phenotype may sit well with habitat in northeastern Kyushu more than the wing type of coastal Kyushu population. However, to test the hypothesis of wing shape selection, functional morphology analyses couple with sequencing of candidate nuclear loci associated with wing venation and wing pattern formation should be condected.

4.4 Comparison of molecular and morphological data

Several studies have adopted wing shape and genetic data comparison. Some of them revealed that the wing shape is as sensitive as molecular data (Miguel *et al.*, 2010; Muñoz-muñoz *et al.*, 2011; Schutze *et al.*, 2012). The results from this thesis demonstrated consistency between evolutionary relationships inferred from wing shape and molecular data (Chapter 2 and 3; Fig. 3-6), although some discordance in the relationships among

populations within subspecies *T. f. fischeri* has been observed. Phylogenetic tree based on mitochondrial and nuclear sequences placed the Tsushima population at the basal part of the Kyushu population clade, whereas phenotypic diagrams placed the Kyushu II and Ainoshima populations at the base instead of the Tsushima population (Fig. 3-6). This discordance may reflect different aspects of the same story. While molecular data reflect older historical gene flow, the morphological data may be detecting subtle differences resulting from more recent gene flow (Schutze *et al.*, 2012). In spite of the difference at population level between phenograms obtained from morphological data and the phylogenetic tree inferred from molecular data, the concordance at the subspecies level suggests the presence of a phylogenetic signal in the wing shape of *T. fischeri*.

4.5 Taxonomy implication

According to the results of both molecular and morphological analyses in this study, taxonomic classification in subspecies level of Japanese *T. fischeri* is suggested. *Tongeia fischeri* in Japan should be classified into three subspecies and one hybrid population: (i) *T. f. japonica* in central Honshu, (ii) *T. f. shojii* in western Honshu and Shikoku, (iii) *T. f. fischeri* in coastal area of Kyushu, Tsushima and adjacent islands, and one hybrid population between *T. f. shojii* and *T. f. fischeri* in northeastern Kyushu (Fig. 3-1). This classification is concordance with the previous classification (Hida, 2005; Satonaka, 2009; Shirôzu, 2006; Yago, 2007), except for the occurrence of hybrid population, which is detected for the first time in this study.

Several wing venation characters were observed during geometric morphometric analysis (Fig. 4-1). In subspecies *T. f. fischeri*, the Sc vein in the forewing of specimens of Kyushu populations did not reach the edge of the wing (black arrow in Fig. 4-2). In Wakayama populations of *T. f. shojii*, the R2 vein in the forewing originates from the R1 vein, rather than from the discal cell as in other population (white arrow in Fig. 4-2). On hindwing, the CU1 and M3 veins of subspecies *T. f. fischeri* usually originate distally to the wing margin and sometime originate far from the discal cell (blue arrow in Fig. 4-2). These characters may be useful and can be used together with wing pattern characters (e.g. in Hida, 2005; Satonaka, 2003) as a diagnostic character to recognize specimens and as an evolutionary character in further systematic or evolutionary studies.

4.6 Future directions

This study opens a door for further investigations. Population genetic studies in particular areas and further sampling would provide a better understanding of the gene flow and structure of the genetic lineages of *T. fischeri*, consequently, would facilitate further investigation phylogeographic pattern of Japanese insects in general. Further investigation of *T. fischeri* and other animals should focus on certain areas, such as an Akaishi mountain range in Central Honshu, Tsushima islands and Kyushu.

Hybrid zone in *T.fischeri* observed here provides a good opportunity to study a role of hybridization in evolution of the butterly. Further sampling, another population genetic marker (e.g. microsatellites), and geographic variation in hostpalnt preference should be investigated for understanding of population history of the butterflies.

High variation in wing venation of *T. fischeri* discovered here is unexpected. It would be interesting to add other morphological traits, such as genital organs, which is supposed to be much diverged in allopatric population. The investigation of genital trait can also be used in evolution study of sex organ in insect, such as testing the lock-and-key hypothesis.

Figure legends

Figure 4-1 Map of Japan at the height of the last glacial maximum (about 20,000 years ago) shows two migration routes of Japanese terrestrial animals (grey arrows). Brown shading shows lands above sea level, blue is sea, white is unvegetated areas covered by snow, and the black outline indicates present-day lands (after Davison *et al.*, 2005).

Figure 4-2 Forewing (upper) and hindwing (lower) of *Tongeia fischeri* showing some diagnostic characters.

Figure 4-1

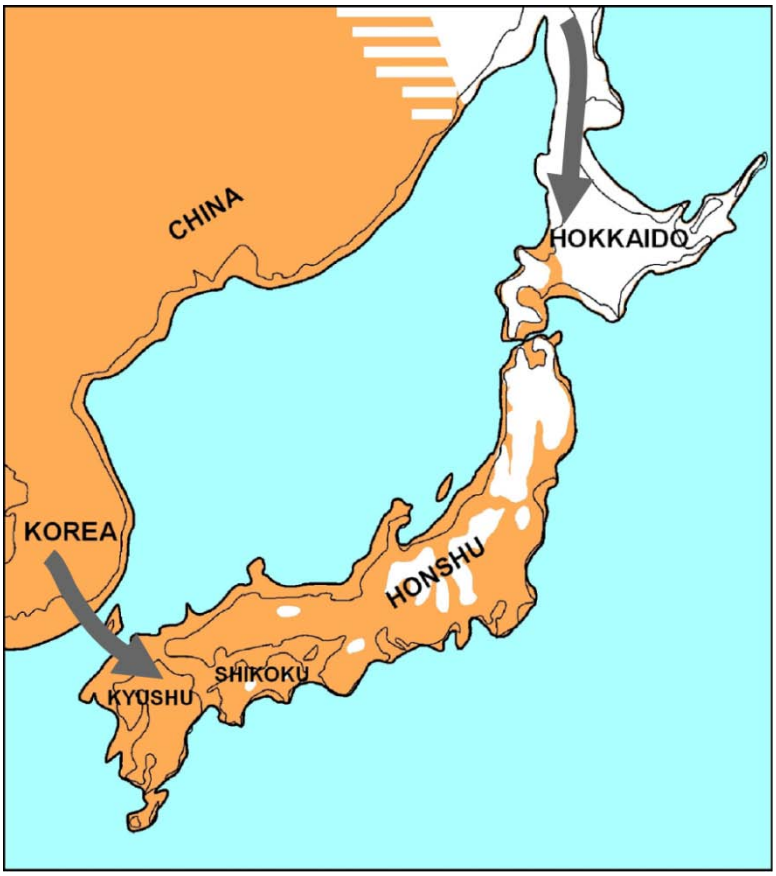
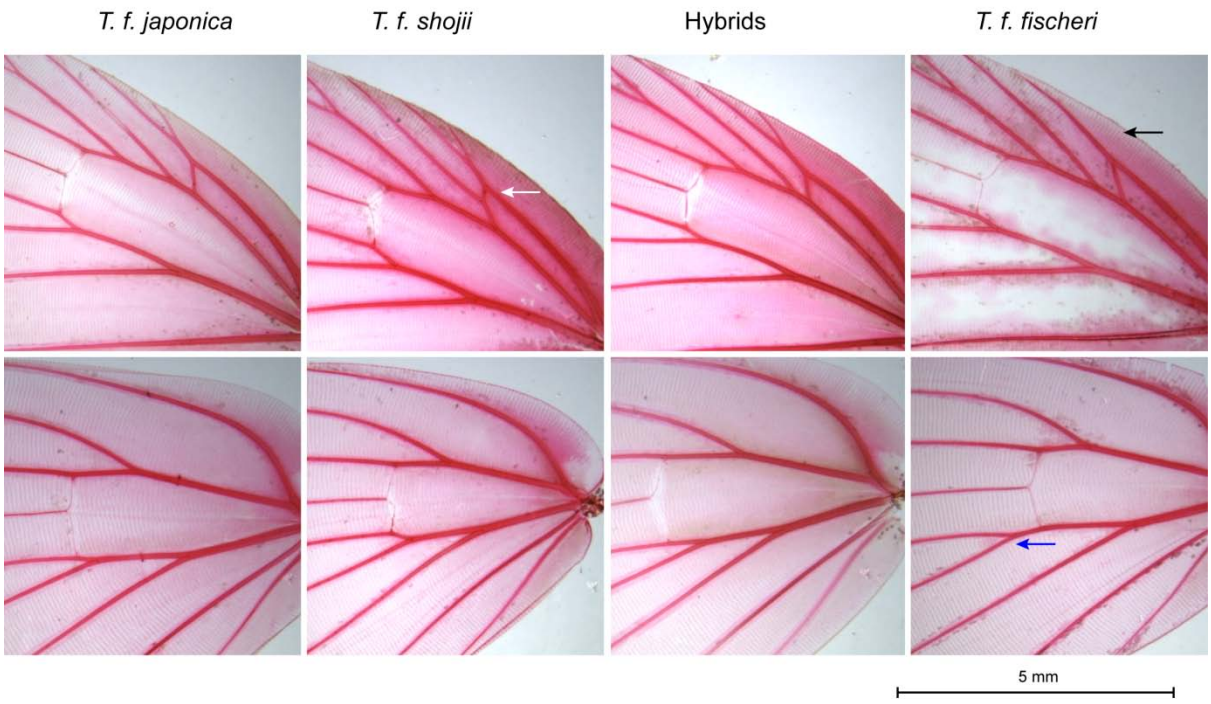


Figure 4-2



ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor, Prof. Tsutomu Hikida, for all of his tremendous support, not only in academic life but also the other suggestions in daily life that made my stay and study in Japan successful with joy. Without his encouragement, I would not have been able to achieve the scholarship for my graduate studies from the Japanese Government, which is extremely important for me as a student coming from a developing country with limited resources. I am forever indebted to him.

This thesis would have not been possible without great assistance from Dr. Masaya Yago, who inspired me with an idea of the project, provided me valuable sources of reference papers and over a hundred of useful materials. I would like to sincerely thank him for all of his help. I am also indebted to Professor Tateo Itoh who donated DNA samples that were used in this study.

Special thanks to N. Hirai, Y. F. Hsu, S. Churkin, H. Hida, J. Geji, T. Mizobe, K. Nishijima, Y. Tôyama, M. Satonaka, A. Mishima, T. Takahachi, Y. Kohara, O. Fukuda, Y. Oshima, K. Yoshida, H. Noshita, Y. Nishiyama, D. Enomoto, Y. Hamaoka, A. Michibata, H. Sugiura, K. Yano, Y. Furuhashi, Y. Kondo, M. Nonaka, Y. Takagi, Y. Miyoshi, K. Niwano, T. Shinkawa, M. Mine, M. Kamigaki, T. Nakatani, and M. Hibino for specimens donation, H. Huang and J. Asahi for valuable taxonomic publications.

Many techniques in molecular biology that I used in this study were a result of kind training by Dr. Takushi Kishida. I would like to thank him for his cooperation. Further appreciation goes to Dr. Mamoru Toda, Dr. Takuya Kiyoshi, Dr. Dai Suzuki, Dr. Yuta Shintaku, Mr. Kazuki Kurita and other members of Laboratory of Systematic Zoology for their help and cheerful laboratory environment. I am also indebted to Mr. Takafumi Nakano for field trips assistance, Ms. Eri Kawaguchi from gCOE for her effort in DNA sequencing, Ms.Teeranee Techasrivichien for proofreading an earlier version of this thesis, and Dr. Elizabeth Nakajima for grammar checking.

I would like to thank Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japanese government for the scholarship and financial support throughout my study. This study was partially supported by the Global COE program for Biodiversity and Evolution (A06) from MEXT, Japan.

Finally, I would like to thank my friends and family for endless support and love that have encouraged me into achieving another success of my life.

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